Herbivore induced changes in the plant's proteome and the role of atypical herbivore-responsive proteins in *Nicotiana attenuata*

Dissertation

Zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. Nat.)

Vorgelegt dem Rat der Biologisch-Pharmazeutischen Fakultät
der Friedrich-Schiller-Universität Jena

von Master of Science in Botany
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1.
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Tag der Doktorprüfung:

Tag der öffentlichen Verteidigung:

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1. Introduction

In order to cope with herbivory, plants must change their physiologies in complicated ways. After herbivory, plants tune their transcriptional responses in a sophisticated manner to produce direct or indirect defenses. The mechanisms of these induced responses can be examined at any stage, starting from genome organization (genomics) and gene expression (transcriptomics) to protein levels (proteomics) to metabolite contents (metabolomics). More recently, large-scale transcriptional analyses with microarrays have broadened the scope of the analysis and revealed coordinated changes in hundreds of transcripts, suggesting that large-scale shifts in metabolism accompany the activation of defense responses (Reymond et al., 2000; Halitschke et al., 2001; Hermsmeier et al., 2001; Schittko et al., 2001; Kessler and Baldwin, 2002; Halitschke et al., 2003; Reymond et al., 2004; Ralph et al., 2006; Ralph et al., 2006). How the changes in the transcriptome translate into changes in the proteome and metabolome that eventually account for the phenotype of increased resistance is unknown, largely because the proteomic changes elicited by herbivore attack remain unstudied. It is becoming increasingly clear that proteomic changes can not be directly predicted from changes in the transcriptome. Furthermore, in a given cellular microenvironment both proteins and transcripts interact with other molecule(s) in specific ways, and these interactions determine the regulation, expression, activity, and the stability of specific mRNA and protein molecules.

Genes regulated during the plant-herbivore interaction have been represented in almost all aspects of metabolism with a substantial fraction coming from primary metabolism (Alignan et al., 2006; Ralph et al., 2006; Schimdt and Baldwin, 2006; Kant and Baldwin, 2007; Tian et al., 2006). While there are many possible reasons for this reconfiguration, primary metabolites could function either directly as defenses or indirectly by influencing an herbivore's ability to detoxify secondary metabolites (Felton et al., 1992; Govenor et al., 1997; Green et al., 2001). With the development of transformation systems and the identification of genes that control secondary metabolite biosynthesis, it is now possible to rigorously test the defensive function of a secondary metabolite by engineering metabolite-deficient plants that otherwise grow normally and examining their performance in native environments with native herbivores (Kessler et al., 2004; Steppuhn et al., 2004; Zavala et al., 2004; Steppuhn and Baldwin, 2007). Testing the potential defensive function of an aspect of primary metabolism is a much greater challenge.

This thesis focuses on the proteomic changes in plants following attack from the specialist herbivore *Manduca sexta* and elicitors which are known to mimic the herbivory responses. Moreover, the roles of seven atypical herbivore-responsive proteins in plant-herbivore interaction were assessed at organismic level. Detailed characterization of two photosynthetic proteins was done by silencing the expression of two photosynthetic proteins in *N. attenuata* plants to explicit the influence of decreased photosynthesis on herbivore resistance.

Nicotiana attenuata as a model system in chemical ecology

Nicotiana attenuata Torr. Ex Wats. (Solanaceae), native of the Great Basin Desert of North America, is a post-fire annual. Dormant seeds respond to a combination of germination stimulants in wood smoke (Baldwin et al., 1994) and inhibitor allelochemicals from the unburned litter (Peterson et al., 2002), and as a consequence synchronously germinate into the nitrogen-rich soils of the post-fire environment. The initially high population densities of this ephemeral pioneer plant decline with the appearance of stronger competitors. Potential herbivores have to recolonize and establish new populations with every generation of plants. Therefore, *N. attenuata* plants encounter a variety of herbivores and pathogens.

N. attenuata is largely self-compatible but has maintained features for out-crossing. Occasionally it is pollinated by hawkmoths (Sime and Baldwin, 2003). Selfing and generation times of 2-3 months predestine this plant for laboratory studies in general and genetic engineering in particular. *N. attenuata* exhibits natural genotypic variation in defense traits, a prerequisite for studying the evolution of these traits. Since it illustrates the point of the previous one Arizona genotype shows no constitutive or jasmonate-induced production of proteinase inhibitors and no herbivore-induced elicitation of the volatile cis-α-bergamontene (Glawe et al., 2003).

N. attenuata is attacked by herbivores from more than 20 different taxa, including mammalian browsers that consume entire plants as well as intracellular feeding insects, and functions also hosts polyphagous and oligophagous organisms. The larvae of leaf-chewing insect herbivores *Manduca sexta* and *Manduca quinquemaculata* (Lepidoptera, Sphingadae) are major defoliators of wild tobacco. Larvae of other leaf-chewing insect herbivores, *Heliothis virescens* and *Spodoptera exigua* (Lepidoptera, Noctuidae), feed similarly but both are polyphagous. Major pests on many crops, they are only occasionally observed on *N. attenuata*.

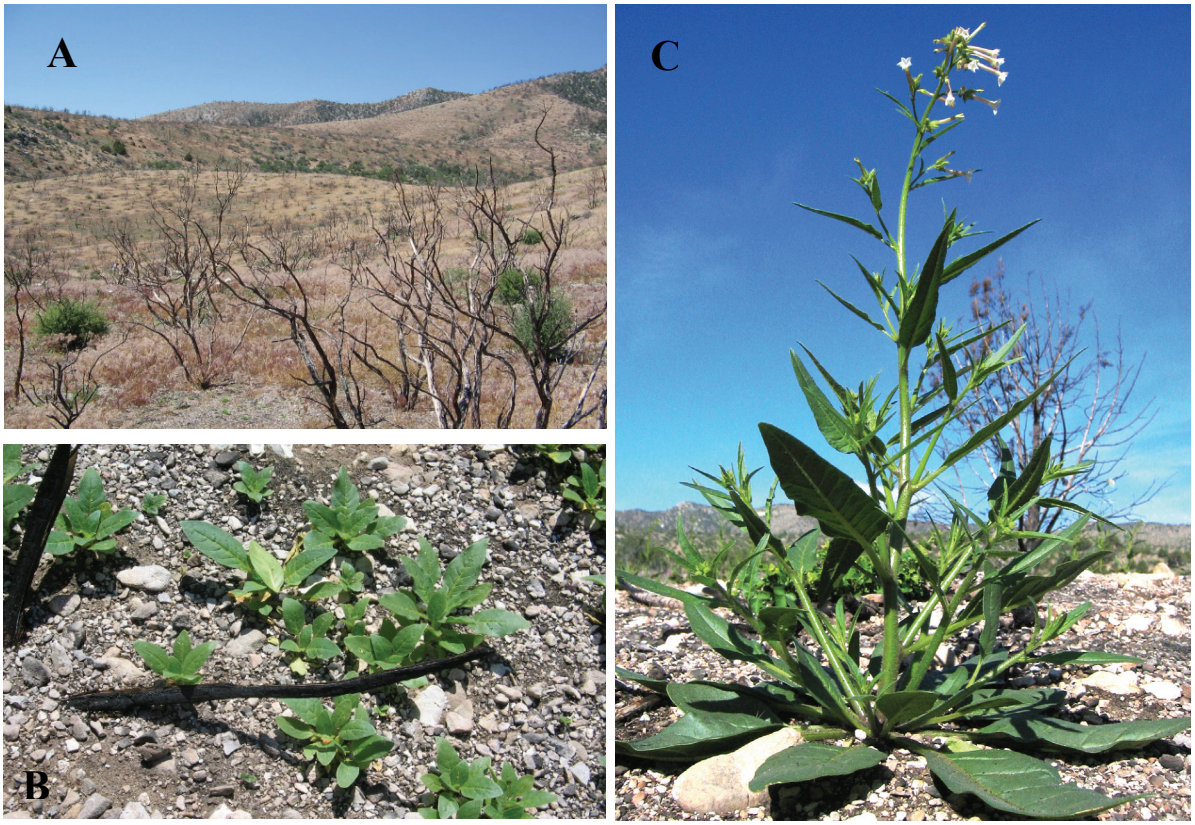

Figure 1. A. Burned field site (Saint George, Utah) **B.** *Nicotiana attenuata* rosettes on burned soil **C.** Elongating and flowering plants – Sources: A-B by Sirsha Mitra; C by Celia Diezel.

Another herbivore, *Tupiocoris notatus* (Heteroptera, Miridae), is a mesophyll feeder and an abundant pest on native plant populations, solanaceous crops (*N. tabacum*, *L. esculentum*), and their wild relatives (*Datura wrightii*, *Solanum carolinense*, *S. viarum*, and *N. rustica*). Phloem-feeders *Myzus nicotianae* and *M. persicae* (Hemiptera, Aphididae) are globally distributed and capable of interbreeding.

How *N. attenuata* reacts after herbivory was discovered by studying the responses of *N. attenuata* after *Manduca sexta* attack. Attacked plants recognize the wound response and the presence of fatty acid amino acid conjugates (FACs) in *M. sexta*'s oral secretions (OS), which is introduced to the wounds during feeding. The reorganization begins with the short-distance mobile signal, which triggers mitogen-activated protein kinases (MAPK). MAPK activation is followed by changes in transcriptional responses and a jasmonate burst and by the long-distance mobile signal defense-related metabolites are produced (Wu et al., 2007).

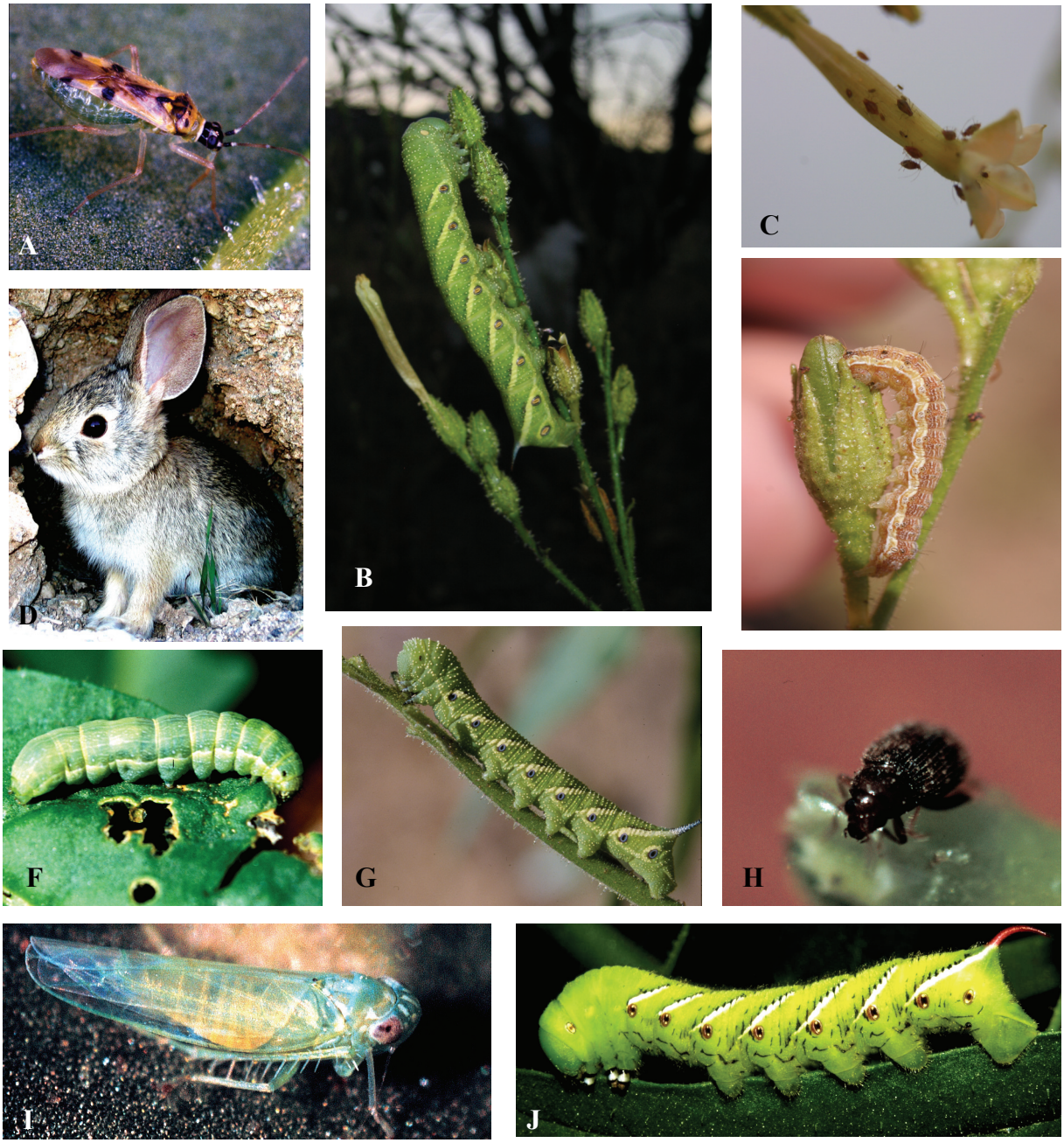

Figure 2. Herbivores of *Nicotiana attenuata*. **A**. *Tupiocoris notatus* (suckfly); **B, G** *Manduca quinquemaculata* (Tomato hornworm); **C** *Myzus nicotianae* (Tobacco aphids); **D** Bunny ; **E** *Heliothis virescens* (Tobacco budworm); **F** *Spodoptera exigua* (beet armyworm); **H** *Epithrix spp*. **I** Emboasca *spp*. **J** *Manduca sexta* (Tobacco hornworm). Sources: A, G, H, I by Andre Kessler; B, C, D, F, J by Danny Kessler; E by Anke Steppuhn.

To study plant-herbivore interaction, a subset of *N. attenuata*'s transcriptome, which had been isolated from plants attacked by *M. sexta* and *Tupiocoris notatus*, was analyzed by differential screening processes, namely differential display RT PCR (DDRT), subtractive libraries, and cDNA amplified-length polymorphism (AFLP) (Hermsmeier et al., 2001; Halitschke et al., 2003; Hui et al., 2003; Voelckel and Baldwin, 2003). Moreover, comparative transcriptional analysis identified several herbivory-induced genes (Voelckel and Baldwin, 2004). However, it is not clear whether these transcriptional changes are translated into proteomic changes.

The objective of this thesis is to reveal the proteomic changes that occur after herbivory by two dimensional gel electrophoresis and a reverse genetics approach, to understand the how proteomic and transcriptomic changes are correlated. Other objectives are to elucidate the functional consequences of atypical herbivore-responsive proteins by silencing their expression in *N. attenuata* plants and to characterize transformed plants. Herbivore attack reduces the levels of photosynthetic proteins and plant's photosynthetic capacity. Therefore the consequences of decreased photosynthesis were analyzed in *N. attenuata* plants by silencing the expression of two major photosynthetic proteins.

Manuscript I describes the changes in *N. attenuata*'s proteome after different elicitation treatments. Differentially expressed proteins were identified with MALDI-TOF and Q-TOF analysis. 17 identified proteins were checked for their m-RNA accumulation. This study reveled that FACs are the main elicitors present in the larval OS and proteomic changes are most often correlated with transcriptomic changes. In general, proteins that increased were involved in primary metabolism, defense, and transcriptional and translational regulation; those that decreased were involved in photosynthesis. Silencing one of the substantially changed photosynthetic proteins, RuBPCase activase, revealed that decreasing this protein reduces photosynthesis and plant biomass but enhances nitrate accumulation.

Manuscript II describes the role of 7 atypical herbivore-responsive proteins at the whole organismic level. The study reveals that candidate proteins are selectively up- and down- regulated after herbivory and apart from their known biochemical functions, play roles in plant-herbivore interaction by influencing the metabolic framework in both plants and insects. In addition, larval midgut protein analysis by 2D-electrophoresis revealed how dynamically the larval proteome responded to the different diets.

Manuscript III describes the influence of decreased photosynthesis on herbivore performance in *N. attenuata*. *N. attenuata* plants were silenced for the expression of their RuBPCase activase (RCA) and RuBPCase (RUB) expression, and characterized for their photosynthesis at different leaf positions, growth, jasmonate (JA) and JA-Ile levels, accumulation of defense metabolites, and resistance to the generalist herbivore *Spodoptera littoralis*. Silencing these two photosynthetic proteins has affected plants' photosynthesis rates and growth similarly but defense metabolite accumulation and herbivore resistance differently.

2. List of Manuscripts and Author's Contributions

Manuscript I

Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. VII. Changes in the plant's proteome

Plant Physiology (2006) **142**: 1621-1641

This manuscript describes the large-scale changes in *N. attenuata* plants after attack from *M. sexta* larvae. Using 2-dimensional gel electrophoresis, MALDI-TOF and LC-MS/MS, we characterized the proteins in phenolic extracts of leaves elicited by larval attack, and/or leaves wounded and treated with OS, FAC-free OS, and synthetic FACs. Here, we characterized 90 proteins which exhibited reproducible elicitor-induced changes. A reverse proteomics approach provided sequence data from 17 elicited proteins that correlated elicited transcript and protein accumulation patterns. We analyzed the function of RuBPCase activase (RCA) by virus-induced gene-silencing (VIGS). The idea of proteomics project was conceived by Ashok Giri, Jorge Zavala, Hendrik Wünsche, and Ian T. Baldwin. 2-Dimenssional gel electrophoresis for wild-type plants was done by me, Hendrik Wünsche and Ashok Giri. Mass spectrometric analyses of proteins were done by Alexander Muck and Aleš Svatoš. The experimental designs of the RCA-silenced plants were developed by me and Ian T. Baldwin. Experimental work, RNA isolation, cDNA synthesis, RT-PCR and cloning of candidate genes, sequence assembly, silencing of RCA by VIGS and protein isolation, 2D-electrophoresis, and functional analysis of RCA-silenced plants were done by me. I wrote part of the introduction, materials and methods, results, and discussion related to the experiments I did. All experiments and the final draft of the manuscript were carried out under the supervision of Ian T. Baldwin.

Manuscript II

Silencing 7 herbivory-regulated proteins in *Nicotiana attenuata* to understand their function in plant-herbivore interactions

Proceedings of the National Academy of Sciences of the United States of America: In review

This manuscript presents both a conceptual breakthrough in how to understand gene function at a whole-plant level, as well as new empirical insights into traits that are involved in the interaction. We were motivated to study the gene function at the organismic level because of the availability of better biochemical expression system. We present an "ask-the-proteome" approach to understand why seven atypical herbivore-responsive proteins are differently regulated in *N. attenuata* when it is attacked by *M. sexta* larvae. The seven proteins, namely, vacuolar ATPase (*Naatpe*), cinnamyl alcohol dehydrogenase (*Nacad*), chaperonin (*Nacpn*), glyceraldehyde 3 phosphate dehydrogenase (*Nagpdh*), glutamine synthase (*Nagmps*), oxygen-evolving protein (*Naoep*), and transketolase (*Natk*), were found to be differentially regulated in our previous proteomic analysis (Giri et al., 2006). Here we attempt to understand why they are regulated because they don't play in any obvious role in herbivore resistance or tolerance. We silence the expression of the genes coding for these proteins by virus-induced gene silencing (VIGS) and analyze the consequences for the plant-insect interaction from the perspective of both the plant (by measuring growth, photosynthetic capacity, and secondary metabolite production) and the insect (by measuring growth, gut protease activity, and midgut proteomic analysis). The experiments were planned by me, Ashok Giri and Ian T. Baldwin. I was responsible for the VIGS-silencing of candidate genes with support from Ashok Giri, Vandana Hivrale and the people listed in the acknowledgments. All other experiments were done by me in close collaboration with Vandana Hivrale and Hendrik Wünsche. I wrote a first draft of the manuscript; the manuscript was a combined effort by me, Hendrik Wünsche, and Ian T. Baldwin.

Manuscript III

The effect of decreased photosynthesis rate on herbivore performance in *Nicotiana attenuata*

Plant Physiology: In preparation

This manuscript presents the effect of decreased photosynthesis on herbivore performance. Two major photosynthetic proteins, RuBPCase activase (RCA) and RuBPCase (RUB), were found to be differentially regulated in our previous proteomic analysis (Giri et al., 2006). Here we attempt to understand how the decrease in photosynthesis influences the resistance of *N. attenuata* to herbivores. We silence the expression of the genes coding for these proteins by agrobacterium-mediated transformation. *RCA*- and *RUB*-silenced plants were screened for their photosynthesis rate and analyzed for growth, defense metabolite production, jasmonate signaling, and resistance to herbivores. The experiments were planned by me and Ian T. Baldwin. Plant transformation was done by S. Kutschbach, A. Wissgott, J. Wurlitzer, and T. Guse. All other experiments were done by me. The manuscript was put together by me and Ian T. Baldwin.

3.1 Manuscript I

Molecular Interactions between the Specialist Herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and Its Natural Host *Nicotiana attenuata*. VII. Changes in the Plant's Proteome

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Plant Physiology 2006, 142: 1621-1641

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Abstract

When *Manduca sexta* attacks *Nicotiana attenuata*, fatty acid amino-acid conjugates (FACs) in the larvae's oral secretions (OS) are introduced into feeding wounds. These FACs trigger a transcriptional response that is similar to the response induced by insect damage. Using 2-DE, MALDI-TOF, and LC-MS/MS, we characterized the proteins in phenolic extracts and in a nuclear fraction of leaves elicited by larval attack, and/or leaves wounded and treated with OS, FAC-free OS, and synthetic FACs. Phenolic extracts yielded ~600 protein spots, many of which were altered by elicitation, whereas nuclear protein fractions yielded ~100 spots, most of which were unchanged by elicitation. Reproducible elicitor-induced changes in 90 spots were characterized. In general, proteins that increased were involved in primary metabolism, defense, transcriptional and translational regulation; those that decreased were involved in photosynthesis. Like the transcriptional defense responses, proteomic changes were strongly elicited by the FACs in OS. A semi-quantitative RT-PCR approach based on obtained peptide sequence was used to compare transcript and protein accumulation patterns for 17 candidate proteins. In 6 cases the patterns of elicited transcript accumulation were consistent with those of elicited protein accumulation. Functional analysis of one of the identified proteins involved in photosynthesis, RuBPCase activase (RCA) was accomplished by virus-induced gene-silencing. Plants with decreased levels of RCA protein had reduced photosynthetic rates, RuBPCase activity and less biomass, responses consistent with those of herbivore-attacked plants. We conclude that the response of the plant's proteome to herbivore elicitation is complex, and integrated transcriptome-proteome-metabolome analysis is required to fully understand this ubiquitous ecological interaction.

Introduction

The majority of studies examining the induced defense responses of plants after insect attack have focused on the dynamics of specific genes, proteins, and metabolites that are thought to be responsible for changes in resistance elicited by attack. More recently large-scale transcriptional analyses with microarrays have broadened the scope of the analysis and revealed coordinated changes in hundreds of transcripts, suggesting that large-scale shifts in metabolism accompany the activation of defense responses (Reymond et al., 2000; Halitschke et al., 2001; Hermsmeier et al., 2001; Schittko et al., 2001; Kessler and Baldwin, 2002; Halitschke et al., 2003; Reymond et al., 2004; Ralph et al., 2006a, 2006b). How the changes in the transcriptome translate into changes in the proteome and metabolome that eventually account for the phenotype of increased resistance is unknown, largely because the proteomic changes elicited by herbivore attack remain unstudied. It is becoming increasingly clear that proteomic changes can not be directly predicted from changes in the transcriptome. Furthermore, in a given cellular microenvironment both proteins and transcripts interact with other molecule(s) in specific ways, and these interactions determine the regulation, expression, activity, and the stability of specific mRNA and protein molecules. Parallel large-scale transcription and protein analyses hold the promise to illuminate the function and regulation of genes and proteins (Bertone and Snyder, 2005; Lange and Ghassemian, 2005; Peck, 2005). Recent developments in mass spectrometric peptide sequencing now allow for protein identification on the proteomic scale (Aebersold and Mann, 2003; Glinski and Weckwerth, 2006). Information obtained with such tools will provide valuable insights into the mechanisms which attacked plants use to shift their metabolic priorities from growth to defense (Walling, 2000; Hahlbrock et al., 2003).

Nicotiana attenuata Torr. Ex Wats. (Solanaceae), a post-fire annual inhabiting the Great Basin Desert, has a number of well-described herbivore-induced direct and indirect defenses (Baldwin, 2001). Direct defenses include nicotine and several other metabolites, and defense proteins such as trypsin proteinase inhibitors (TPIs) and threonine deaminase (TD) (Hermsmeier et al., 2001; Schittko et al., 2001; Kang et al., 2006). Indirect defenses comprise the terpenoid and green leaf volatile alarm signals that provide information about the activity and location of feeding herbivores to the community of predators (Kessler and Baldwin, 2002). Several such combinations of direct and indirect defenses appear to be especially effective in reducing pest populations in an evolutionarily stable manner (Baldwin, 2001).

The detailed information gained from such biological systems may lead to the development of valuable tools for managing insect infestation of crops more wisely (Anderson et al., 2005).

High-throughput transcriptional analysis of the interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *N. attenuata* identified a large-scale transcriptional reconfiguration, which entailed decreases in photosynthetic-related processes and increases in defense-related processes (Walling, 2000; Halitschke et al., 2001, 2003; Hui et al., 2003). The standardized and synchronized treatment of elicited responses, namely, the application of *M. sexta* oral secretions (OS) to leaves punctured by a pattern wheel, a treatment which was found to mimic the transcriptional and metabolic responses of *M. sexta* larvae attack (Schittko et al., 2001; Halitschke et al., 2003; Hui et al., 2003; Roda et al., 2004), is crucial for understanding plant-herbivore interactions. The major constituents in OS from *M. sexta* and several other lepidopteran insects responsible for the differential activation of *N. attenuata* genes have been identified as fatty acid-amino acid conjugates (FACs). More than 70% of the OS-elicited transcriptional changes in *N. attenuata's* wound response as determined by microarray analysis can be mimicked by introducing chemically synthesized FACs into puncture wounds (Schittko et al., 2001; Halitschke et al., 2003; Hui et al., 2003; Roda et al., 2004).

As a follow-up to transcriptional analysis, we undertook a comparative proteome analysis of the *M. sexta-N. attenuata* interaction. Proteomic analysis was carried out by comparing the patterns of leaf proteins in the leaves of undamaged plants to those in elicited and attacked plants by two-dimensional gel electrophoresis (2DE). We performed two types of proteomic analysis, addressing two main questions: First, how does a plant respond to the different elicitors found in *M. sexta* OS? To answer this question we compared the patterns of protein accumulation when punctured wounds were treated with OS by those when the puncture wounds were treated with water, FACs, OS which had their FACs removed by ion-exchange chromatography (OS-FAC-free), and *M. sexta* larval feeding. Second, how do these responses change over the time when leaves are known to increase their resistance to insect attack? To answer this question, we measured the accumulation of identified proteins at 6, 12, 30, 48, and 72 h after OS-treatment of puncture wounds. We used a RT-PCR approach to determine the association between candidate proteins showing differential accumulation patterns and the abundance of their encoding mRNAs. In addition, functional analysis of one of the identified proteins involved in photosynthesis, RuBPCase activase (RCA) was accomplished by gene silencing. This study identifies several well-characterized proteins

whose direct and indirect roles in insect-elicited responses were not previously known, as well as several proteins of unknown function.

RESULTS

Comparison of 2DE protein profiles of control and elicited leaves of *N. attenuata*

Source leaves +1, +2, and +3 of rosette-stage plants of *N. attenuata* were punctured parallel to the mid-vein with a fabric pattern wheel six times at 30-min intervals (**Fig. 1**). To induce the plants with different elicitors, various solutions were applied to the punctured leaves (W+OS, W+FAC, and W+OS-FAC-free) or larvae were released on these leaves (**Fig. 1**). The leaf proteins were extracted with different methods and analyzed.

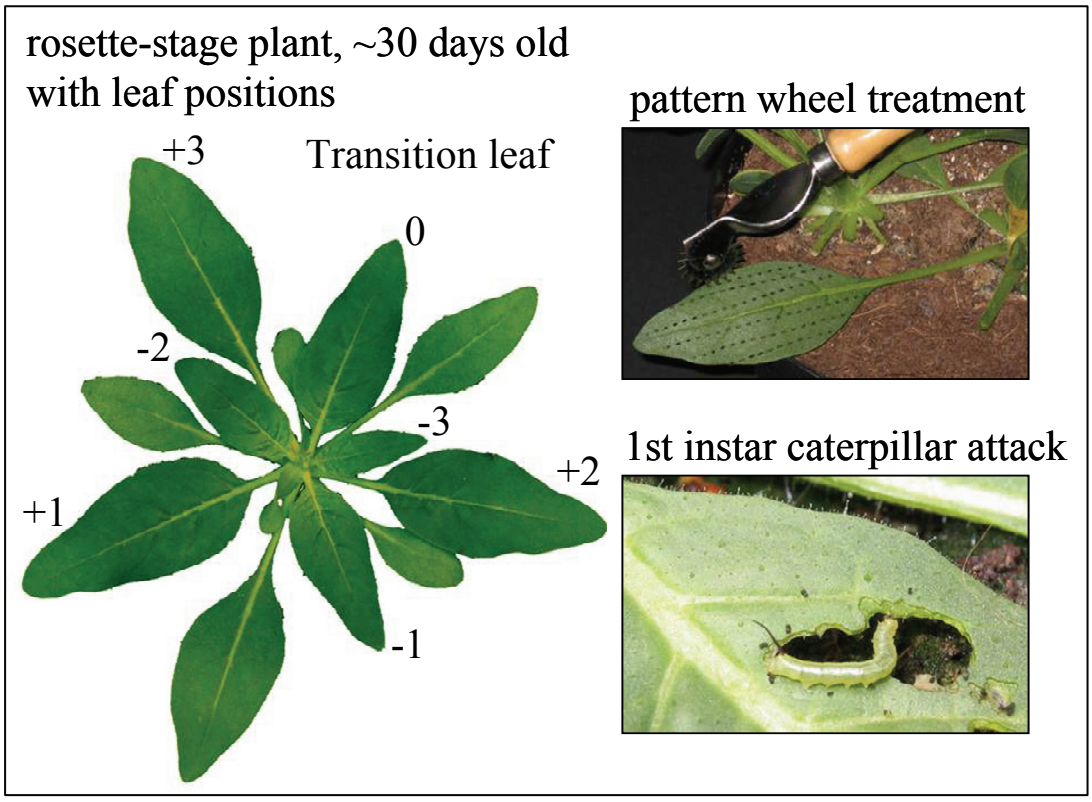


Figure 1: Leaf numbering and elicitation procedures. The left panel depicts the numbering system of leaf nodes of 30-day-old rosette-stage plants, and the upper right panel the leaf-wounding procedure with the pattern wheel, according to which three leaves +1, +2 and +3 were wounded and elicitor solutions applied six times at 30-minutes interval. The elicitor solutions were water, 0.005% Triton X-100, *M. sexta* OS, OS without fatty acid amino acid conjugates, FACs (OS-FAC-free), and chemically synthesized FACs; and in the lower right panel a feeding first-instar *M. sexta* larva.

Phenolic extracts of *N. attenuata* leaf proteins yielded ~600 protein spots on a 2D-SDS-PAGE. Protein spots exhibiting variations in extracts from control and elicited leaves were identified and compared among three to five biological replicates. One such biological replicate represents 2 to 4 plants and 3 elicited or adjacent un-elicited (systemic) leaves on each plant. Representative 2DE gel images of leaf proteins from the 30 h harvest of control and W+OS elicited leaves are presented in **Figure 2.** Each protein preparation was analyzed on at least 3 parallel 2DE gels. In total we generated and analyzed 72 gels for phenol extract (three biological replicates, six treatments, and five time points) and 14 gels for nuclear extract (three biological replicates and two time points). Analysis of the nuclear protein fractions revealed ~100 protein spots with no significant variation between control and OS-elicited leaf extracts (**Fig. S1**). Since the total leaf protein profiles (phenolic extracts) responded to elicitation, while the nuclear protein fraction did not, we focused our efforts on characterizing the elicited changes in the total protein extracts. The similar protein patterns observed in control leaves from five harvests removed at 6 to 72 h after elicitation indicated that the developmental or leaf maturation-related changes that occurred during this time did not significantly influence the part of the proteome that was detectable in our analysis (**Figs. 2, S2,** and **S3**). In contrast, we identified 90 spots that exhibited differential accumulation patterns from 6 to 72 h after OS elicitation when wounded leaves were compared with the respective control leaves. These proteins were successfully identified using peptide mass fingerprints and/or peptide sequencing (**Table I**). Among these 90 proteins, 35 showed consistent accumulation trends in induced leaves 30 h after OS elicitation (**Fig. 2** and **Table II**). Changes in these proteins were meticulously compared in four induction treatments (**Table II**). Fifty-one other proteins identified in this study, which showed consistent increased or decreased accumulation patterns at specific harvest times in at least two replicates, were identified. The MS data of 4 proteins did not yield information for the database search and *de novo* sequencing.

Identification of proteins by MALDI-TOF and LC-MS/MS

The tryptic peptide mass fingerprints of selected proteins were determined from MALDI-TOF spectra using the green plant database of the National Centre for Biotechnology Information (NCBI; http://www.ncim.nlm.nih.gov); protein spots showing identity with RuBPCase were omitted from further analysis. Peptide sequence data obtained from LC-MS/MS were compared with green plant or non-redundant NCBI databases. Amino acid

sequences from de novo data with unspecified protein hits were searched using MS-BLAST. Most of the forty-five proteins identified by peptide sequences agree with the peptide mass fingerprints. An additional 42 proteins have been identified by their peptide mass fingerprints, which match at least 4 peptides. Detailed information obtained from these 84 protein spots, such as identification scores, number of matching peptides, and peptide sequences, and identification of their putative function with accession numbers of database proteins, molecular masses, and isoelectric points are provided in **Table I**. More than 20% of proteins remain unidentified due to the lack of a database match or a match only to proteins with unknown functions.

Patterns of protein accumulation elicited by the different treatments

Accumulation patterns for 35 proteins were monitored in the four elicitation treatments and compared to patterns observed in control leaves. Differences among these treatments were seen in only a few spots. The larval feeding treatment was terminated after 48 h and the responses in this treatment were compared with those of the W+OS treatments. A similar but lower rate of protein accumulation was found in the larval treatment compared to the W+OS treatment, perhaps because the amount of damage and the amount of OS introduced into wounds after 48 h of feeding by 5 neonate larvae is likely less than inflicted by the W+OS treatment. Of the 35 differentially regulated proteins in these two treatments, 37% (13 proteins) were commonly regulated (9 up- and 4 down-regulated) **(Fig. 3A; Table II)**. Interestingly, spot 83, a chloroplast glutamine synthetase, was up-regulated in caterpillar-attacked leaves but was not regulated in the other elicitation treatments (**Table II**). However, a vacuolar H ATPase A2 subunit, threonine deaminase, and one spot with glyceraldehydes-3-phosphate dehydrogenase of caterpillar-attacked leaves share accumulation patterns similar to those of leaves treated with OS.

Because the amount of mechanical damage and elicitor solutions introduced represented parameters that did not differ among the W+OS, W+FAC, and W+OS-FAC-free treatments, the cause of the differences is more easily inferred. Of the 35 proteins that were differentially regulated by these three treatments, 12 (7 up- and 5 down-regulated) were commonly regulated among all three treatments (**Fig. 3B; Table II**). The seven commonly up-regulated protein spots were RNA-binding protein (spot 24)**,** acyl CoA synthetase 7 (spot 32), S-adenosylmethionine synthetase (spot 34), O-acetylserine thiol lyase (spot 43), RCA (spot 46), spermidine synthase (spot 50), and potassium sodium symporter (spot 57).

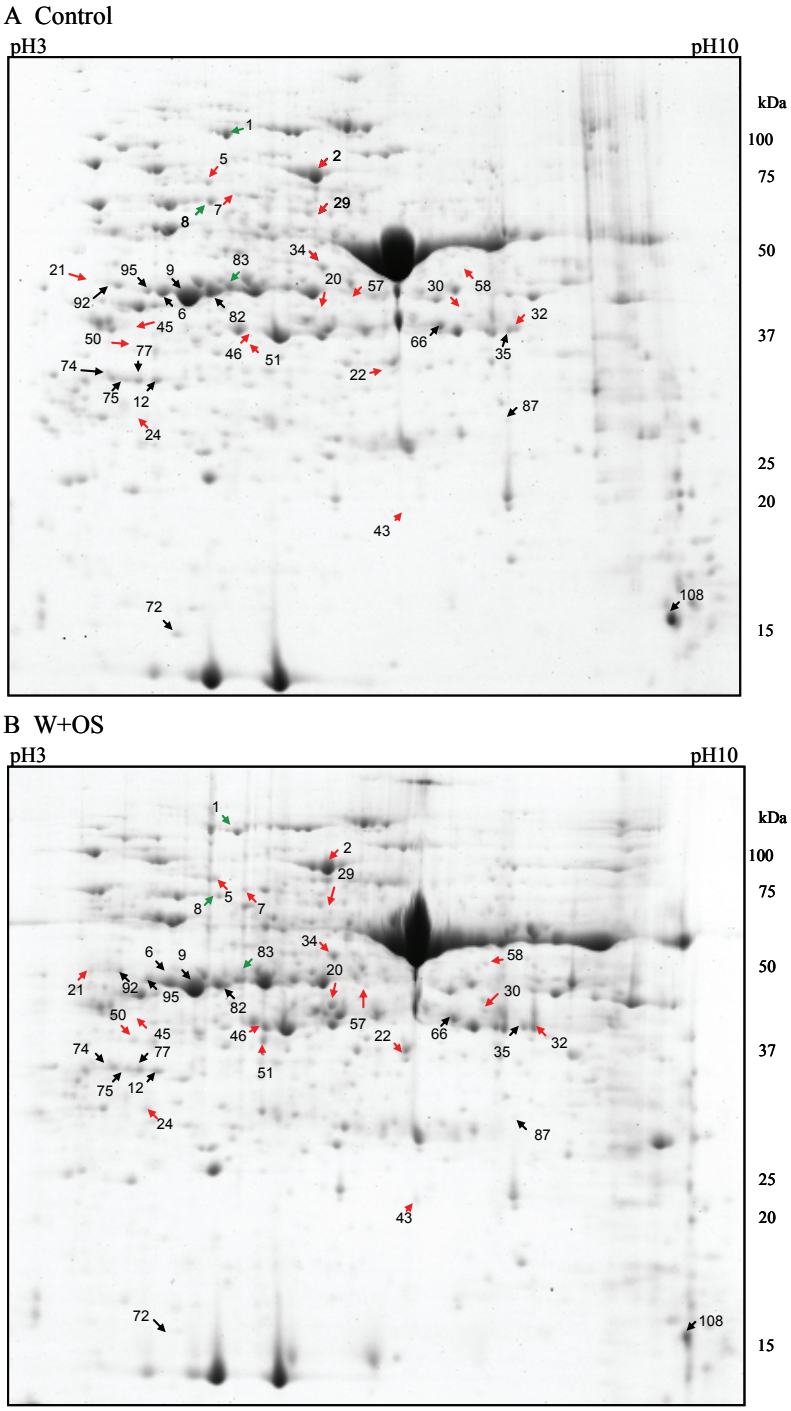


Figure 2: **Proteomic maps of two-dimensionally separated control and elicited leaf proteins.** Locally treated leaves were collected 30 h after the first OS elicitation and proteins were extracted using the phenol extraction protocol. Approximately 550 µg of protein was resolved on 24 cm IPG strips of pH range 3-10 NL (isoelectric focusing), further separated on 12% acrylamide SDS-PAGE gels, and stained with Bio-Safe Coomassie G-250 stain. Gels were scanned and images analyzed using PDQuest software. Representative protein profiles (from 9 gels) of control **(A)** and W+OS-elicited **(B)** leaves from different plants were used to compare protein accumulation patterns. Numbers and arrows indicate protein spots experiencing up- (red arrows) or down- (black arrows) regulation, or no change (green arrows) in response to OS elicitation at this time point. For identities of the protein spots and their accumulation patterns in response to several elicitation treatments at from 6 to 72 h time points, see Tables I and II.

Interestingly, four of the five down-regulated spots identified as RCA consistently accumulated in lower amounts (spots 6, 9, 82, and 95) and the fifth protein is oxygen-evolving enhancer protein 1 (spot 75).In order to determine which proteins were regulated by the FACs in OS, we compared the regulation elicited by the W+OS and W+FAC treatments. Seven proteins were commonly regulated and no proteins were uniquely regulated by the FAC treatments that were not changed by the W+OS treatment. Only 1 spot (OSJNBa0039c07.4, spot 1) was uniquely regulated by W+OS-FAC-free treatment, and 1 spot (Quinone oxidoredcutase-like protein, spot 8) was commonly regulated by this treatment and the W+FAC treatment, demonstrating that FACs account for the majority of the elicitor activity of OS at the protein level. However, OS clearly contain factors other than FACs that elicit differential accumulations of proteins, as can be seen by comparing the responses elicited by the W+OS and W+OS-FAC-free treatments (**Fig. 3B**). These treatments commonly regulated the following five proteins which were not regulated by the W+FAC treatment: RCA (spots 20 and 51), transketolase (spot 2), the oxygen-evolving enhancer protein 1 (spot 12), and the glycine-rich RNA-binding protein (spot 72). These results demonstrate that although FACs are the main elicitors of specific changes in protein accumulation in OS, other factors are clearly also active.

Table I: Protein spots identified with MALDI-TOF and/or LC-MS/MS. Mass spectrometric identification of protein spots with differential accumulation after OS elicitation using comparison of the measured sequences or peptide mass fingerprints with the NCBI green plant protein database.

Spot #	Accession #	Name of protein	Protein identification data					Response
			MALDI Score/# pep	LC-MS/MS Score/# pep	Theoretical kD/pI	Observed kD/pI	Peptide sequence (MS/MS)	
1	XP_472335	OSJNBa0039C07.4 protein	9.7/18	12.71/1	98.4/5.98	87.2/5.79	(K)TAIAEGLAQR(I)	II
2	S58083	Transketolase	9.79/12	12.07/6	75/6	72.3/7.05	(R)FLAIDAVEK(A) (K)NGNTGYDEIR(A) (K)VTTTIGFGSPNK(A) (K)ANSYSVHGSGLGAK(E) (K)ALPTYTPESPADATR(N) (R)KTPSILALSR(Q)	III
4	BAA77604	Plastidic aldolase	5.69/4	12.77/3	42.5/7.48	35.4/7.23	(K)YTGEGESEEAK(K) (R)SAAYYQQGAR(F) (K)YTGEGESEEAKK(G)	No change
5	AAO23981	Vacuolar H^+-ATPase A2 subunit	8.71/11	10.28/2	68.6/5.37	68.7/5.54	(R)NLEDETR(-) (R)SGDVYIPR(G)	I
6	Q40565	RuBPCase activase	10.13/12	11.62/5	48.3/8.17	40.3/5.02	(R)TDNVPEEAVIK(I) (R)VYDDEVRK(W) (K)WVSGTGIEAIGDK(L) (K)LLNSFDGPPTFEQPK(M) (R)VQLAETYLK(E)	II
7	AAG59585	Threonine deaminase	12.43/10	12.72/6	65.6/6.21	64.5/5.94	(R)VYDVAIDSPLQNAAK(L) (R)EDMQSVFSFK(L) (K)DVYDEGR(N) (K)QALVLYSVGVNTK(S) (K)SSQLNTVNLTNNNLVK(E) (K)FLDAFSPR(W)	I

Table I. (Continued from previous page)

Spot #	Accession #	Name of protein	Protein identification data					Response
			MALDI Score/# pep	LC-MS/MS Score/# pep	Theoretical kD/pI	Observed kD/pI	Peptide sequence (MS/MS)	
8	AAM62737	Quinone oxidoreductase like protein		12.77/1	32.7/6.08	63/5.56	(K)VVAAGLNPVDAKR(R)	III
	AAB39828	Chaperonine		11.51/1	18.10/10.2		(K)IVNDGVTVAR(E)	
9	Q40565	RuBisCO activase		11.62/11	48.3/8.17	39.1/5.25	(K)GLVQDFSDDQQDIAR(G) (R)QYNMDNTLDGFYIAPSFMDK(L) (K)VPLILGMWGGK(G) (R)KMGINPIMMSAGELESGNAGEPAK(L) (K)MGINPIMMSAGELESGNAGEPAK(L) (R)VYDDEVR(K) (R)KWMSGTGIEAIGDK(L) (K)LLNSFDGPPTFEQPK(M) (K)LLEYGNMLVQEQENVKR(V) (K)RVQLAETYLK(E) (K)EAALGDANADAINTGNF(-)	II
12	2002393A	Oxygen evolving complex protein	5.17/4	11.46/3	26.48/4.9	29.8/4.83	(R)RLTFDEIQSK(T) (K)FCLEPTSFTVK(A) (R)GGSTGYDNAVALPAGGR(G)	II
13	AAL71857	Dehydroascorbate reductase	4.07/4		23.6/8.2	22.8/8.3		I
14	BAB64833	Phosphoglycerate mutase	6.02/7	10.4/2	61/6.3	63.7/6.17	(K)LVDLALASGK(I) (R)LDQVQLLLK(G)	III
16	AAP04394	Glutathione S-transferase		12.69/1	1/4.07	23.6/8.37	(-)LLDVYETR(-)	I
17	Q42961	Phosphoglycerate kinase precursor	5.2/12		50.1/8.75	41.6/6.99		V

Spot #	Accession #	Name of protein	Protein identification data					Response
			MALDI	LC-MS/MS	Theoretical	Observed		
			Score/# pep	Score/# pep	kD/pI	kD/pI	Peptide sequence (MS/MS)	
18	NP_199203	Myosin heavy chain MYA2	1.9/4		169.8/7.9	20.5/8.08		I
19	BAA77604	Plastidic aldolase	5.71/5		42.5/7.4	35.9/6.37		V
		Unspecified		12.36/1	1.4/6.93		(-)GAVQLWKYLEDR(-)	
20	Q40565	RuBisCO activase	8.95/16	14.54/7	48.3/8.4	37.8/7.19	(K)LVVHITK(N) (K)SFQCELVFR(K) (R)EAAEIIR(K) (R)EAAEIIRK(G) (R)TDNVPEEAVIK(I) (R)VYDDEVRK(W) (R)VQLAETYLK(E)	I
21	AAT38067	Putative glycoprotease	1.2/4		40.8/6.19	45.1/<4.55		I
		Unspecified		12.72/1	0.9/11.7		(-)VVKKKGFR(-)	
22	AAS90324	3 dehydroquinate dehydratase	5.31/4		56.1/6.08	32.4/7.93		I
		Unspecified		12.72/1	1.1/9.71		(-)NAGANAYAALTK(-)	
23	BAD28273	Hypothetical protein	5.38/4		22.2/9.7	35.3/>9.3		IV
24	AAM97013	Unknown protein Arabidopsis thaliana	7.37/5		113.5/9.06	26.6/4.67		I
	BAD32968	RNA binding protein-like		12.77/1	35.6/10.4		(R)YADPVQR(E)	

Table I. (Continued from previous page)

Spot #	Accession #	Name of protein	Protein identification data					Response
			MALDI Score/# pep	LC-MS/MS Score/# pep	Theoretical kD/pI	Observed kD/pI	Peptide sequence (MS/MS)	
25	Q9M5M6	60S ribosomal protein L30	3.62/4		12.2/9.6	87.2/8.48		IV
		Putative subtilase		11.6/1	1.2/9.71		(-)TVNTVGAGAATYK(-)	
27		Unspecified		12.72/1	1.5/8.89	23.9/4.8	(-)LKPDPNTCGGKDFK(-)	IV
28	S72477	Probable cinnamyl alcohol dehydrogenase		12.72/1	38.3/5.86	38.9/8.51	(R)DTSGVLSPFNFSR(R)	I
29	AAB33843	Aldehyde dehydrogenase	4.95/4		52.6/5.64	55/7.14		I
30	CAA79702	Mitochondrial formate dehydrogenase precursor	8.54/7	11.59/6	41.8/7.08	37/8.5	(K)IVGVFYK(A) (K)TVGTVGAGR(I) (K)MDSELENQIGAK(F) (K)FEEDLDK(M) (K)GVLIVNNAR(G) (K)DGELAPQYR(-)	I
32	AAM28874	Acyl CoA synthetase 7	4.53/4		77.2/6.51	35.3/8.89		IV
34	P43282	S-adenosylmethionine synthetase	8.4/4	11.38 /2	42.6/6.07	46.3/7.14	(K)SVVASGLAR(R) (K)TQVTVEYK(N)	I
35	CAF22093	Glyceraldehyde 3 phosphate dehydrogenase		12.21/2	19.59/8.82	35/8.84	(R)AASFNIIPSSTGAAK(A) (K)VVISAPSK(D)	II
37	BAD52936	Putative Homocysteine S methyltransferase 4	4.73/5		34.5/6.09	17.9/8.85		IV

Table I. (Continued from previous page)

Spot #	Accession #	Name of protein	Protein identification data					
			MALDI Score/# pep	LC-MS/MS Score/# pep	Theoretical kD/pI	Observed kD/pI	Peptide sequence (MS/MS)	Response
		Unspecified		12.72/1	1.2/6.75		(-)QLMD AATTTNAK(-)	
38	AAM65239	Unknown protein	5.74/4		32.3/10.1	32.6/7.96		V
	1SM4A	Ferredoxin Nadp Reductase		11.22/2	33.3/6		(R)LYSIASSAIGDFGDSK(T) (K)DNTFVYMCGLK(G)	
39	NP_054979	Hypothetical protein SpolCp075	2.32/4		7/10.8	35.3/>9.3		I
40	AAF27063	F4N2.23	4.38/4		95.3/6.22	37.6/5.68		V
42	AAM46780	Latex plastidic aldolase	6.51/4		42.6/8.6	35.1/5.91		V
43	BAA01279	O-acetylserine thiol lyase	3.89/4		34.1/5.61	19.6/8.08		I
45	CAA75381	Translation elongation factor	5.34/10	13.28/1	27.2/5.09	42/4.64	(R)NTTVTGVEMFQK(I)	I
46	Q40565	RuBisCO activase	7.82/13	12.72/13	34.36/6.76	35.1/6.14	(K)IVVHITK(N) (K)SFQCELVFR(K) (K)SFQCELVFRK(M) (R)YREAAEIIR(K) (R)EAAEIIRK(G) (K)FYWAPTR(E) (R)VYDDEVR(K) (R)VYDDEVRK(W) (K)WVSGTGIEAIGDK(L) (K)LLNSFDGPPTFEQPK(M) (K)LLEYGNMLVQEQENVK(R) (K)LLEYGNMLVQEQENVKR(V) (R)VQLAETYLK(E)	I

Table I. (Continued from previous page)

Spot #	Accession #	Name of protein	Protein identification data					Response
			MALDI Score/# pep	LC-MS/MS Score/# pep	Theoretical kD/pI	Observed kD/pI	Peptide sequence (MS/MS)	
47	P49319	Catalase isozyme 1 Salicylic acid binding protein	5.15/18		56.7/7.02	54.9/8.64		V
48	AAD39604	F23M19.3	2.69/4		67/9.5	73.2/9.09		V
49	CAD27635	Maturase	4.22/4		61.2/9.68	24.5/>9.3		IV
50	BAA24535	Spermidine synthase	5.81/8	-13.9/1	34.4/5.3	35/4.55	(K)ASFCLPSFAK(R)	I
51	CAA78703	RuBisCO activase	8.99/8	14.54/6	48.3/8.4	33/6.16	(K)SFQCELVFR(K) (R)TDNVPEEAVIK(I) (R)VYDDEVR(K) (K)LLNSFDGPPTFEQPK(M) (R)VQLAETYLK(E) (K)EAALGDANADAINTGNF(-)	I
53	CAE05740	OSJNBb0017I01.20	1.68/4		40.8/5.7	24.5/>9.3		IV
54	AAG40343	Hydroxymethyltransferase	5.94/4		51.7/7.6	55/8.73		IV
55	NP_918618	OSJNBa0094H06.18	2.33/4		5.6/10.1	22/>9.3		IV
57	AAF97728	Potassium-sodium symporter	2.52/4		61.9/9.13	38.9/7.53		I
58	NP_915483	P0446B05.25	5.04/4		38.6/9.6	42.1/8.5		I
60	XP_470450	Unknown protein	3.86/4		48.2/10.5	37/7.37		I

Table I. (Continued from previous page)

Spot #	Accession #	Name of protein	Protein identification data					Response
			MALDI Score/# pep	LC-MS/MS Score/# pep	Theoretical kD/pI	Observed kD/pI	Peptide sequence (MS/MS)	
61	T07032	S-2-hydroxy acid oxidase	6.05/7		31.2/9.57	44.5/>9.3		I
62	XP_468473	Putative cytochrome P450	5.2/4		58.1/9.4	41.7/5.89		V
64	BAD03386	Hypothetical protein	3.33/4		20.8/10	107.3/<4.5		IV
65	CAA96433	Pectin methylesterase	5.1/4	11.46/1	20.1/9.29	37/8.31	(R)VGADMSVINR(C)	IV
66	CAC80376	Glyceraldehyde 3 phosphate dehydrogenase	5.81/4	12.71/5	34/5.56	36.2/8.33	(K)TLLFGEK(S) (K)KVVISAPSK(D) (R)AASFNIIPSSTGAAK(A) (K)EASYDDIK(A) (K)AAIKEESEGK(L)	II
67	AAN40027	Hypothetical protein	3.48/5		64.7/9.09	37.9/<4.5		V
68	P29790	ATP synthase gamma subunit	9.47/7		41.4/8.4	35/8.64		IV
	CAB39974	Glyceraldehyde 3 phosphate dehydrogenase		12.72/5	34.4/6.15		(K)DAPMFVVGVNEK(E) (R)AASFNIIPSSTGAAK(A) (K)VLPSLNGK(L) (K)EATYDEIK(A) (K)AAIKEESEGK(L)	
69	AAF03099	NADP dependent glyceraldehyde phosphate dehydrogenase		11.71/1	11.8/10.8	31/7.93	(K)DSPLDVIAINDTGGVK(Q)	IV
70	AAG23799	Glyceraldehyde 3 phosphate dehydrogenase		12.66/3	18.2/6.1	35.1/8.73	(R)AAALNIVPTSTGAAK(A) (K)AVALVLPSLK(G) (K)TFAEEVNAAFR(E)	V

Table I. (Continued from previous page)

Spot #	Accession #	Name of protein	Protein identification data					Response
			MALDI Score/# pep	LC-MS/MS Score/# pep	Theoretical kD/pI	Observed kD/pI	Peptide sequence (MS/MS)	
71	AAM62933	Putative chloroplast 50S ribosomal protein L6		14.54/4	24.6/10.2	17.4/>9.3	(R)TLTDNMVVGVSK(G) (R)ITVSGYDK(S) (K)SEIGQFAATVR(K) (K)YADEIVR(R)	V
72	2119042	Glycine rich RNA binding protein	10.74/9	14.54/3	15.6/5.62	15/5.11	(R)NITVNEAQSR(G) (R)EGGYGGGGGYGGGR(R) (R)EGGYGGGGGGGYGGGR(R)	II
74	P81665	Oxygen evolving enhancer protein 1	11.12/9	12.72/2	26.39/4.31	30.2/<4.55	(R)GSSFLDPK(G) (R)GGSTGYDNAVALPAGGR(G)	II
75	P81665_1	Oxygen evolving enhancer protein		12.49/1	1.09/4.07	30.2/<4.55	(K)NAPPEFQK(T)	II
77	T45856	Hypothetical protein F3A4.110	4.26/4		57.2/9.46	30/<4.55		V
	AAP03871	Oxygen evolving complex 33 kDa photosystem II protein		12.71/6	35.17/5.4		(K)RLTFDEIQSK(T) (R)LTFDEIQSK(T) (K)FCLEPTSFTVK(A) (K)DGIDYAAVTVQLPGGER(V) (R)GGSTGYDNAVALPAGGR(G) (R)GDEEELEKENVK(N)	
79	B24430	Glyceraldehyde 3 phosphate dehydrogenase	6.51/8	10.85/4	41.78/8.56	41.6/8.45	(K)YDSMLGTFK(A) (K)IVDNETISVDGK(H) (K)GTMTTTHSYTGDQR(L) (R)AAALNIVPTSTGAAK(A)	V
80	AAR96006	4 - nitrophenylphospha tase like protein		11.68/1	16.5/5.38	29.6/5.18	(R)LVFVTNNSTK(S)	V
82	1909374A	RuBisCO activase	7.01/11		42.7/5.57	41/5.59		II

Table I. (Continued from previous page)

Spot #	Accession #	Name of protein	Protein identification data					Response
			MALDI Score/# pep	LC-MS/MS Score/# pep	Theoretical kD/pI	Observed kD/pI	Peptide sequence (MS/MS)	
83	AAO62992	Chloroplast glutamine synthetase		12.73/2	20.9/5.82	41.7/5.89	(K)AAQIFSDSK(V) (R)DISDAHYK(A)	V
84	AAA34111	RuBisCO	7.71/4		10.1/5.36	14.8/6.37		V
85	AAG14029	NADH dehydrogenase	3.86/4		37.4/9.69	27/>9.3		II
87	BAA31510	Ribosomal protein L4 chloroplast	2.85/4		31.2/6.59	27.6/8.71		II
89	BAD35631	Putative zinc finger and C2 domain protein	3.56/4		35.6/6.47	30.9/>9.3		II
90	AAM64724	Unknown protein	3.22/4		61.7/5.23	87.9/<4.5		V
91	BAD15634	Mitochondrial transcription termination factor like	3.65/4		42.6/9.9	35.9/6.37		V
92	CAB87677	Putative protein	4.78/5		34.7/6.56	42.2/<4.5		II
93	AAR20278	Maturase	6.32/5		55.1/10.4	42.9/4.55		V
94	AAO32311	Putative chloroplast 50S ribosomal protein L6	5.29/6		24.9/10.3	18.9/>9.3		III

Table I. (Continued from previous page)

Spot #	Accession #	Name of protein	Protein identification data					Response
			MALDI Score/# pep	LC-MS/MS Score/# pep	Theoretical kD/pI	Observed kD/pI	Peptide sequence (MS/MS)	
95	CAA78703	RuBisCO activase	9.14/12	11.62/6	48.3/8.4	41.3/4.71	(K)GLVQDFSDDQQDIAR(G) (R)EAAEIIR(K) (R)TDNVPEEAVIK(I) (R)VYDDEVR(K) (K)WVSGTGIEAIGDK(L) (R)VQLAETYLK(E)	II
97	NP_174644	Hypothetical protein	2.74/4		21.6/7.7	19.7/<4.5		V
98	BAD28931	Hypothetical protein	2.39/5		7.4/10.3	39.7/8.6		V
99	AAF76363	I box binding factor	2.77/4		23.9/8.31	42.7/>9.3		V
100	BAD53039	Hypothetical protein	2.88/4		7.3/6.47	41/8.73		V
101	XP_466540	putative speckle type POZ	4.05/8		38.8/10.5	76.2/5.25		V
103	P29302	Photosystem I chain II D2 precursor	5.71/5		22.4/10.1	41.5/9		III
105	CAA71589	Protein g5bf	3.82/4	42.6/3.82	37/8.48			III
108	AAU03361	PS2 Oxygen evolving complex protein 3		14.51/1	0.95/10.9	15/>9.3	(-)GKLKGSGKGK(-)	II
109	O23787	Thiazole biosynthetic enzyme precursor	9.53/11	11.62/1	37.5/5.2	32.9/4.8	(K)ALDMNSAEDAIVR(L)	IV

Table II: Changes of proteins in *N. attenuata* leaves upon several induction treatments. The accumulations of *N. attenuata* leaf proteins exhibiting consistent up- or down-regulation (indicated by arrows) at a minimum of three time points (6 to 72 h) after OS treatment are listed with their p-values of water control (W+W), fatty-acid-amino-acid-conjugates (W+FAC) induction, OS-without-FACs (W+OS-FAC-free), and caterpillar-feeding induction (Larvae) at 48 h. The protein names represent the database identification of the mass spectrometric analyses with the highest confidence. Numbers indicate p-values, n/a (p-value is not available), n/c (no change, spot quantities are equal), x (spot not detectable).

Spot #	Accession #	Name of protein	Induction pattern and *p* values for the treatments				
			W+W	W+OS	W+FAC	W+OS-FAC-free	Larvae
Photosynthesis and photorespiration related metabolism							
6	CAA78703	RuBisCO activase	- 0.14	↓ 0.19	↓ 0.93	↓ 0.11	↓ 0.07
9	XP_473501, Q40565	OSJNBb0078D11.3, RuBisCO activase	- 0.74	↓ 0.04	↓ 0.21	↓ 0.003	↓ 0.05
12	Q40459	Oxygen evolving enhancer protein 1	- 0.31	↓ 0.31	- 0.24	↓ 0.45	- 0.12
20	CAA78703	RuBisCO activase	- n/c	↑ qltv	- n/c	↑ 0.18	- n/c
46	CAA78703	RuBisCO activase	↑ 0.01	↑ qltv	↑ 0.05	↑ 0.15	↑ 0.07
51	CAA78703	RuBisCO activase	↑ 0.02	↑ qltv	- 0.17	↑ 0.18	↑ 0.19
74	P81665	Oxygen evolving enhancer protein 1	- 0.45	↓ 0.05	↓ 0.11	- 0.92	↑ 0.06
75	P81665_1	Oxygen evolving enhancer protein 1	- 0.64	↓ 0.11	↓ 0.15	↓ 0.62	- 0.22
77	AAP03871	Oxygen evolving complex 33 kDa photosystem II protein	- 0.45	↓ 0.05	↓ 0.12	- 0.71	- 0.4
82	1909374A	RuBisCO activase	- 0.43	↓ 0.27	↓ 0.58	↓ 0.02	↓ 0.12
95	CAA78703	RuBisCO activase	↑ 0.06	↓ 0.27	↓ 0.41	↓ 0.02	- 0.29
108	AAU03361	Oxygen evolving complex protein 3	- 0.98	↓ 0.01	- 0.92	- 0.68	- 0.25
Primary metabolism							
1	XP_472335	OSJNBa0039C07.4	- 0.4	- 0.88	- 0.31	↑ 0.09	↑ 0.15
2	S58083	Transketolase	↑ 0.04	↑ 0.77	- 0.32	↑ 0.18	- 0.43
5	AAO23981	Vacuolar H^+-ATPase A2 subunit	- 0.89	↑ 0.13	↑ 0.08	- 0.95	↑ 0.02
7	AAG59585	Threonine deaminase	↑ 0.17	↑ 0.24	↑ 0.34	- 0.67	↑ 0.02
21	AAT38067	Putative glycoprotease	- n/c	↑ qltv	- 0.37	- n/c	- n/c
29	AAB33843	Aldehyde dehydrogenase	- 0.27	↑ 0.12	↑ 0.28	x n/a	- 0.42
30	CAA79702	Mitochondrial formate dehydrogenase precursor	- 0.83	↑ 0.35	↑ 0.18	- 0.49	- 0.9
32	AAM28874	Acyl CoA synthetase 7	- 0.34	↑ 0.22	↑ 0.27	↑ n/a	↑ n/a
34	AAO85809	S-adenosylmethionine synthetase	- 0.73	↑ 0.25	↑ 0.06	↑ 0.11	↑ 0.03
35	CAF22093	Glyceraldehyde 3 phosphate dehydrogenase	- 0.77	↓ 0.8	- 0.36	- n/a	↑ 0.004
43	BAA01279	O-acetylserine thiol lyase	- 0.37	↑ qltv	↑ 0.11	↑ 0.23	↑ 0.18
50	BAA24535	Spermidine synthase	↑ 0.09	↑ 0.08	↑ 0.01	↑ n/a	- 0.34
66	BAC87864	Glyceraldehyde 3 phosphate dehydrogenase	- 0.85	↓ 0.26	- 0.08	- 0.85	↓ 0.08
83	AAO62992	Chloroplast glutamine synthetase	- 0.33	- 0.38	- 0.83	- 0.22	↑ 0.03
Transcription and Translation							
45	BAA02028	Translation elongation factor	- n/c	↑ qltv	- n/c	- n/c	- n/c

Spot #	Accession #	Name of protein	Induction pattern and *p* values for the treatments				
			W+W	W+OS	W+FAC	W+OS-FAC-free	Larvae
72	BAA03741	Glycine-rich RNA-binding protein	- 0.85	↓ 0.02	- 0.21	↓ 0.03	- 0.51
87	BAA31510	Ribosomal protein L4 chloroplast	- 0.13	↓ qltv	- 0.43	- n/a	- n/a
Secretory pathway							
57	AAF97728	Potassium-sodium symporter	↑ 0.08	↑ 0.11	↑ 0.04	↑ 0.008	- n/a
Secondary metabolism							
8	AAM62737	Quinone oxidoreductase-like protein	- 0.99	- 0.76	↓ 0.37	↓ 0.005	↓ 0.21
22	AAS90324	3-dehydroquinate dehydratase	↑ 0.21	↑ 0.002	- 0.43	- 0.83	↑ 0.77
24	BAD32968	RNA-binding-like protein-	↑ 0.18	↑ 0.05	↑ 0.02	↑ 0.04	↑ 0.003
Unspecified function and unknown proteins							
58	NP_915483	P0446B05.25	- 0.64	↑ 0.08	↑ 0.25	- 0.19	- 0.22
92	CAB87677	Putative protein	- 0.39	↓ 0.04	- 0.78	- 0.54	- 0.46

Changes in the patterns of W+OS-elicited protein accumulation

The early (6, 12, and 30 h) and later (48 to 72 h) changes in the W+OS-elicited proteome were characterized by the relative densitometric quantitation of regulated protein spots (**Table I; Figs. 4, S2,** and **S3**). Of these regulated protein spots, 17 are involved in photosynthesis and photorespiration -- 28 in primary metabolism, 13 in transcription and translation processes -- and 9 function in secondary metabolism pathways; one protein in each case was identified in signal transduction, secretory pathway, and cytoskeleton formation (**Table I, Fig. 2**). Twenty proteins were not identified or were identified but had unknown functions. To simplify the analysis, we categorized the protein accumulation patterns as: (I) consistently high accumulation, (II) consistently low accumulation, (III) early high accumulation (up to 30 h) and no change or low later (30 h onwards), (IV) no change or low early on and high accumulation or no change later, and (V) no consistent pattern (**Table I**). Twenty-seven proteins exhibited a type I response (**Table I**). Of these, four proteins belong to the photosynthesis and photorespiration category and eight to the primary metabolism category; four proteins were categorized as involved in secondary metabolism, seven as unknown proteins; one is a translation elongation factor; one protein contributes to the cytoskeleton and one to the secretory pathway. Fifteen proteins exhibited a type II response and are present in the following categories: eight are related to photosynthesis and photorespiration, four to primary metabolism, one to transcription and translation, one to signal transduction, and one is a putative protein. For type I and II responses, we also included proteins which showed consistent accumulation in four harvests. We categorized six proteins with type III responses. One is involved in photosynthesis, three in primary metabolism, one in transcription and translation, and one in secondary metabolism. Sixteen proteins exhibited type IV responses, seven in primary metabolism, four in transcription and translation, and five with no known function. We identified 26 proteins with type V responses, indicating they either have very specific kinetics or were not detected at other harvests of several replicates. In summary, we observed most proteins with similar accumulation patterns during the five time harvests after application of OS to *N. attenuata* leaves. However, few proteins specific to one or two harvest times and exhibiting increased or decreased accumulation patterns were also identified.

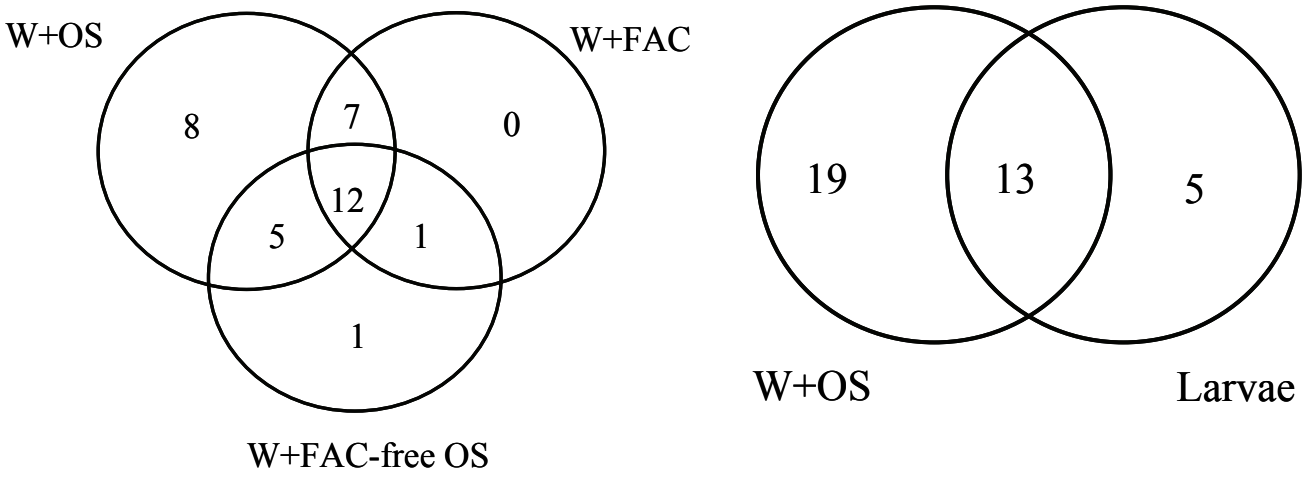


Figure 3: Comparison of protein accumulation patterns in *N. attenuata* leaves among different inducers. The Venn diagram presents the number of protein spots that exhibit differential accumulation patterns among different elicitor treatments compared to control leaves. Shown are a comparison between larvae and W+OS induced leaves **(A)** and a comparison among the W+OS, W+FACs, and W+ OS-FAC-free **(B)**. The numbers in circles indicate the protein spots having differential accumulation and the numbers in the common area represent protein spots with similar patterns of accumulation among the treatments.

The response of RCA protein(s) to OS elicitation

The changes in RCA protein spots were one of the most apparent OS-elicited responses, so much so that these spots reliably differentiated gels of control and elicited samples. We identified seven protein spots that have one or several peptides exhibiting homology with RCA (**Table I and II**). In four spots, MW and pI are very similar in terms of reported RCA proteins from several plant species. After W+OS in elicited leaves compared to controls, the levels of these proteins (spots 6, 9, 82, and 95) decreased (**Fig. 5**), whereas levels of RCA (spots 20, 46, and 51) strongly increased. Since these changes were not significant in W+W or W+FAC treatments, we infer that factors other than FACs in OS are responsible (**Fig. 5D**). This inference is supported by the observation that the responses observed in the W+OS-FAC-free treatment were similar to those observed in the W+OS treatment (**Fig. 5F**). Peptide data obtained from spots 6 and 95 match data from the C-terminal region of RCA protein, whereas spots 9, 20, and 46 yielded peptide sequences spanning almost the entire length of RCA. Protein spot 51 generated peptides from the N-terminal region of RCA. These results suggest that massive changes are occurring in RCA proteins due to proteolytic cleavage, differential subunit formation, or gene expression. RCA is a member of the

chaperonin protein family, which is involved in RuBPCase activation, and RuBPCase is known to be transcriptionally down-regulated after elicitation (Hermsmeier et al. 2001).

To determine whether the changes observed in RCA proteins are due to the proteolytic activity of OS fraction, we analyzed the 2DE protein patterns of undamaged systemic leaves on elicited plants. Interestingly, proteins in spots 46 and 51 were detectable in OS-elicited systemic leaves, whereas decreasing amounts of protein in spots 6, 9, 82, and 95 were noticed in the systemic leaves compared to leaves growing at the same nodes on control plants (**Fig. 5G** and **H**). Furthermore, to confirm that these changes in RCA spots are not due to a plant protein fraction present in OS, we performed a 2DE analysis of OS. Of the nearly 100 proteins we identified by peptide mass fingerprints, no protein spots were detected with similarity to RCA (results not shown). These results demonstrate that even if proteolysis of RCA is responsible for these changes, it occurs via plant metabolism and is not due to the proteolytic activity of OS.

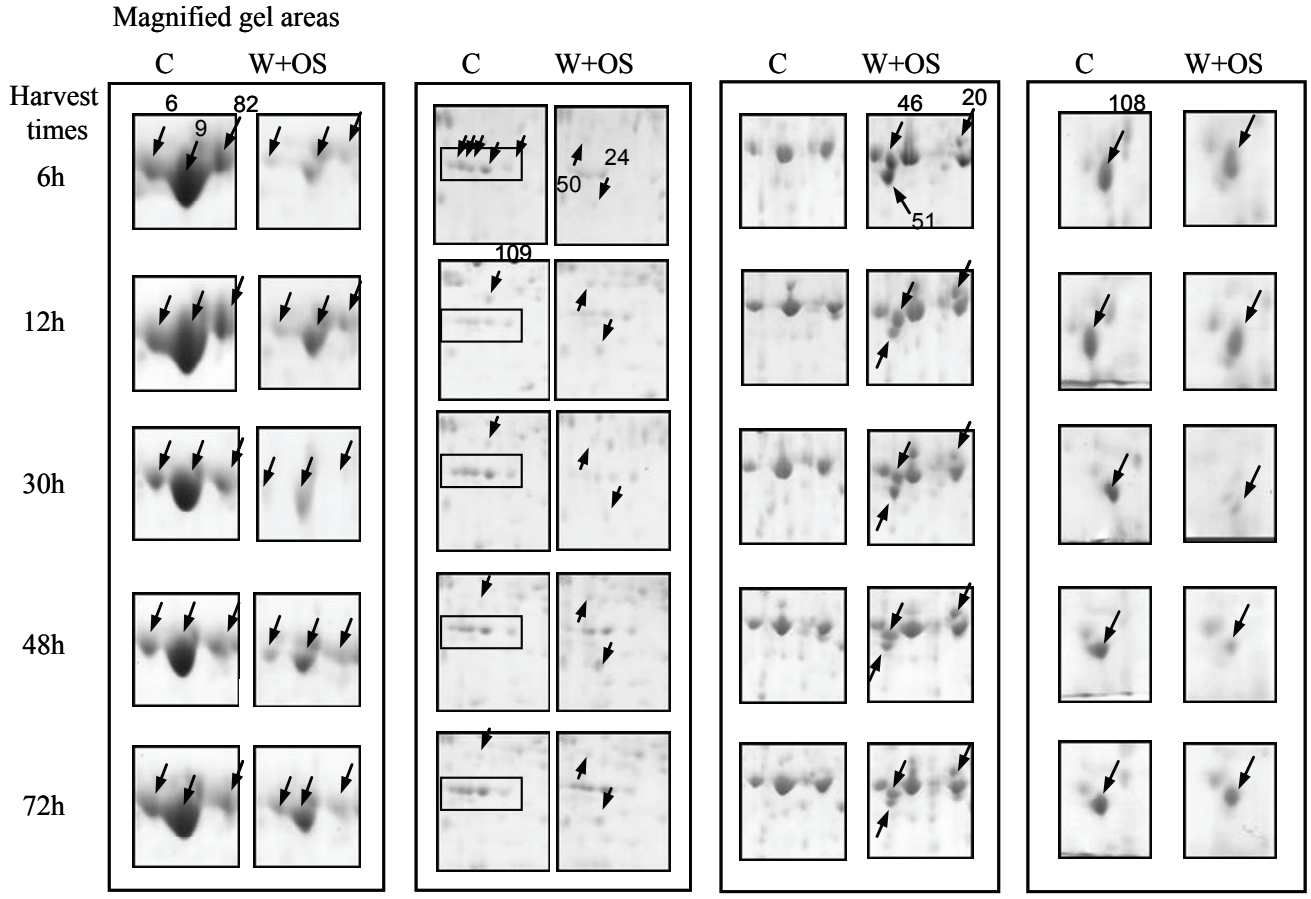


Figure 4: Magnified areas of two-dimensional gels. The 4 panels show magnified areas of control and W+OS elicited leaves at different times after elicitation (6 to 72 h). The boxes in gel area (column 2) encompass spots 74, 75, 77, 78, and 80 (left to right). Numbers of protein spots with arrows are indicated in the first gel of their appearance, whereas the positions of protein spots are shown with arrows in subsequent gels. Proteins eluted from these spots were identified by MALDI-TOF and/or LC-MS/MS (see Table I).

Transcript accumulation patterns in *N. attenuata* elicited leaves

RT-PCR was used to quantify the transcripts of 17 genes coding for 17 differentially accumulated proteins to assess the correlations between protein and mRNA accumulation patterns (**Fig. 6 and 7**). Different peptide sequences obtained from protein spots were used to design primers to compare mRNA accumulation in W+OS elicited leaves with that in control leaves 30 h after elicitation. The well-characterized insect-induced protein TPI was not identified in the protein gels; however, it was used as a positive control. TPI may not be detected in induced leaves in proteomic analysis due to its extractability and/or low levels. Patterns of mRNA accumulation were compared among 17 candidate genes 30 h after the different elicitation treatments (**Fig. 6)**. Levels of mRNA increased after W+OS, W+FAC, W+OS-FAC-free and larval treatments in seven transcripts, namely, *tpi*, *td*, *pgam* (phosphoglycerate mutase), *sams* (s-adenosylmethionine synthetase), *pme* (pectin methyl esterase), *gpdh* (glyceraldehyde3 phosphate dehydrogenase), *fdh* (mitochondrial formate dehydrogenase), and *atpe* (vacuolar ATPase); however, treatment-specific increases were also observed (**Fig. 6A**). *tpi*, *td*, and *sams* transcripts responded maximally to larval feeding, whereas *pgam* and *atpe* accumulated maximally in response to the W+ OS-FAC-free treatment. The W+FAC treatment elicited the largest response in *pme* mRNA. *Ald* (aldolase) transcripts decreased in response to the W+OS and larval treatments, but increased after the W+FAC and W+OS-FAC-free treatments. Levels of *tef* (translation elongation factor) increased after W+OS-FAC-free treatments compared to other elicitor treatments. Glutamine synthase transcripts increased only after W+FAC and W+OS-FAC-free and larval treatments but not after W+OS treatment. Levels of *grp* (glycine-rich RNA-binding protein) and *cpn* (chaperonin) increased after the W+FAC and W+OS-FAC-free treatments, but decreased in response to the presence of larvae. *grp* also showed increased accumulation after W+OS treatments. Levels of *tk* (transketolase) increased maximally after W+OS-FAC-free treatment. Transcripts of *rca* decreased after W+OS treatments and increased after larval and W+OS-FAC-free treatments but remained unchanged after the W+FAC treatment (**Fig. 6B**). Transcript accumulation patterns of seven candidates correlated with their respective protein abundances. The specific mRNA accumulation patterns observed in OS-FAC-free elicited *N. attenuata* leaves indicates that signaling molecule(s) other than FACs exist in *M. sexta* OS, and these also elicit specific protein accumulation patterns.

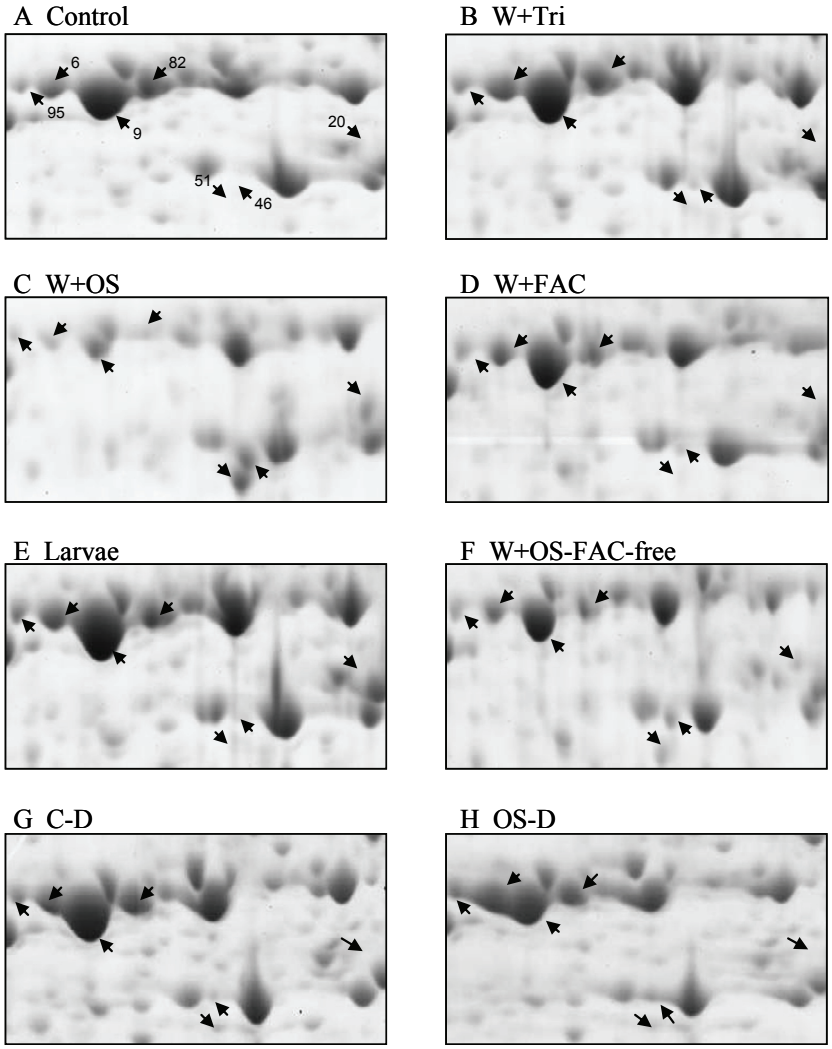
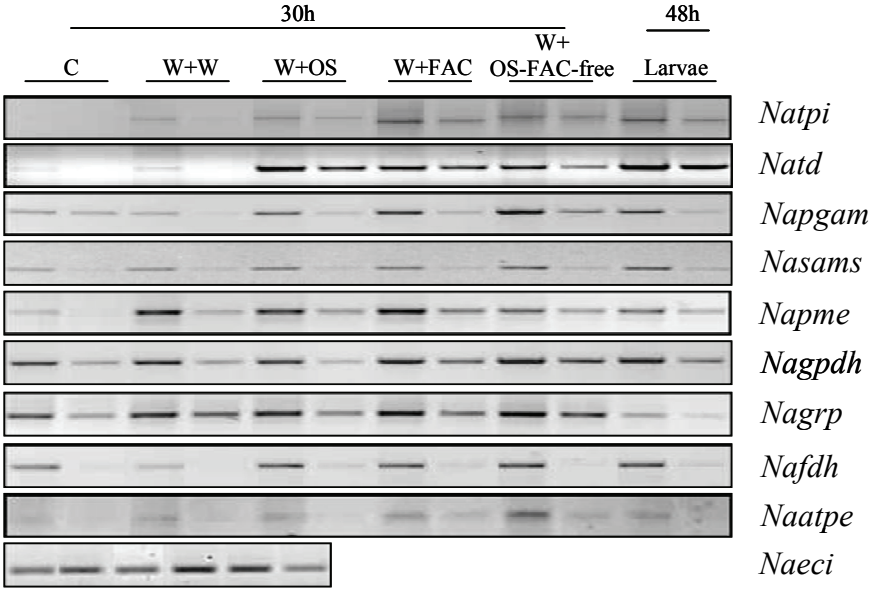


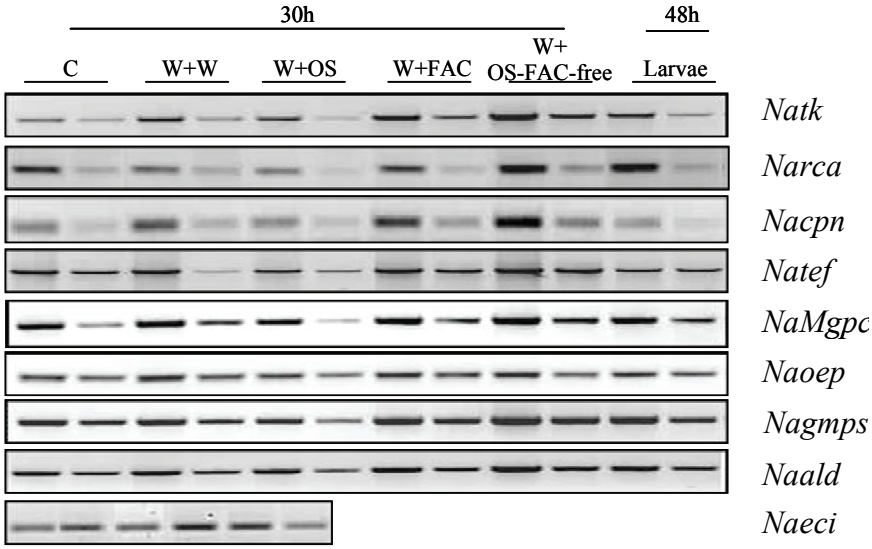
Figure 5: Magnified areas (see areas I and III in Supplementary Figure 4) of two-dimensional gels of protein extracts from control and elicited plants from treated and distal leaves for RCA spots. Leaf protein profiles of control **(A)**, and punctured leaves treated with 0.0025% triton X-100 (W+Tri) **(B),** solutions of *Manduca sexta* OS (W+OS) **(C)**, OS without FACs (W+OS-FAC-free) **(D)**, chemically synthesized FACs (W+FAC) **(E)**, feeding *M. sexta* larva **(F)**, a control of distal leaves (C-D) **(G)** and W+OS of distal leaves (OS-D) **(H)**. Leaves were collected 30 h after the application of elicitor solution and 48 h after the release of larvae on local leaves. The accumulation patterns of RCA spots were compared among these treatments.

The differential accumulation of mRNA was observed in all 17 genes. To simplify the data we categorized accumulation patterns as we had done for the proteins (**Fig. 7**). In control plants, levels of *tpi* transcripts were barely detectable, but they were strongly elicited -- a type I pattern -- by W+OS treatment. This pattern was also found in *td* transcripts where both (*tpi* and *td*) had reached their peaks at 12 h and decreased slightly thereafter. Pectin methyl esterase (*pme*) transcripts were up-regulated at all time points (type I). mRNAs of *pgam*, *sams*, *fdh*, and *atpe* were consistently up-regulated up to 30 h, slightly down-regulated at 48 h, and unchanged at 72 h (type III) (**Fig. 7A**). Transcripts of *gpdh* were slightly up-regulated at 6 h, down-regulated at 12 and 30 h, and up-regulated again at 48 and 72 h (type V).

A



B



6: Gene expression analysis using the RT-PCR approach on candidate proteins differentially elicited in *N. attenuata* leaves after various elicitor treatments. Quantitative RT- PCR analysis of seventeen candidate genes in non-wounded and in wounded leaves treated with different elicitors, which show **(A)** pattern of 9 genes: trypsin protease inhibitor (*Natpi*), threonin deaminase (*Natd*), phosphoglycerate mutase (*Napgam*), s-adenosyl methionine synthetase (*Nasams*), pectine methyl esterase (*Napme*), glyceraldehyde- 3-phosphate dehydrogenase (*Nagpdh*), glycine-rich RNA binding protein (*Nagrp*), mitochondrial formate dehydrogenase (*Namfd*), and ATPase (*Naatpe*); and **(B)** pattern of 8 genes: transketolase (*Natk*), RuBPCase activase (*Narca*), chaperonin (*Nacpn*), translation elongation factor (*Natef*), Mg protoporphyrin IX chelatase (*NaMgpc*), oxygen-evolving protein (*Naoep*), glutamine synthase (*Nagmps*), and aldolase (*Naald*). Transcript abundance were checked after 48 h of larval feeding and 30 h for control (C), and the application of wounding with water (W+W), larval oral secretions (W+OS), fatty acid amino acid conjugates (W+FAC), and OS devoid of FACs (W+OS-FAC-free). PCR reactions were carried out with two concentrations of cDNA for all primers, in at least 3 replicates. A single concentration of cDNA was used for ECI (AB010717, sulfite reductase) primers.ECI used as internal standard to determine the equal amplification of cDNA. The gene names, accession numbers, primer sequences, size of amplified cDNA fragments, and respective peptide sequence maps of proteins are provided in Supplemental Table I and Supplemental Figure 5.

g*rp* accumulated fewer transcripts in induced leaves at early compared to later time points (30 to 72 h) (type IV). Levels of *tk*, *rca*, *tef*, *mgpc* (Mg-protoporphyrin IX chelatase), *ald* and *cpn* (6-48 h), showed decreased mRNA accumulation at all time points (type II) (**Fig. 7B**). Transcripts of *gmps* (glutamine synthatase) and *oep* (oxygen-evolving protein) were down-regulated consistently up to 30 h, but at 48 h the levels of accumulation were the same as in control leaves; at 72 h after OS application (type V) the levels were down-regulated. In summary, mRNA accumulation patterns match protein accumulation patterns in several candidate proteins; however, cases in which protein and mRNA accumulation did not match need to be studied further. One possible explanation for such discrepancy might be due to the presence of several isoforms of these proteins.

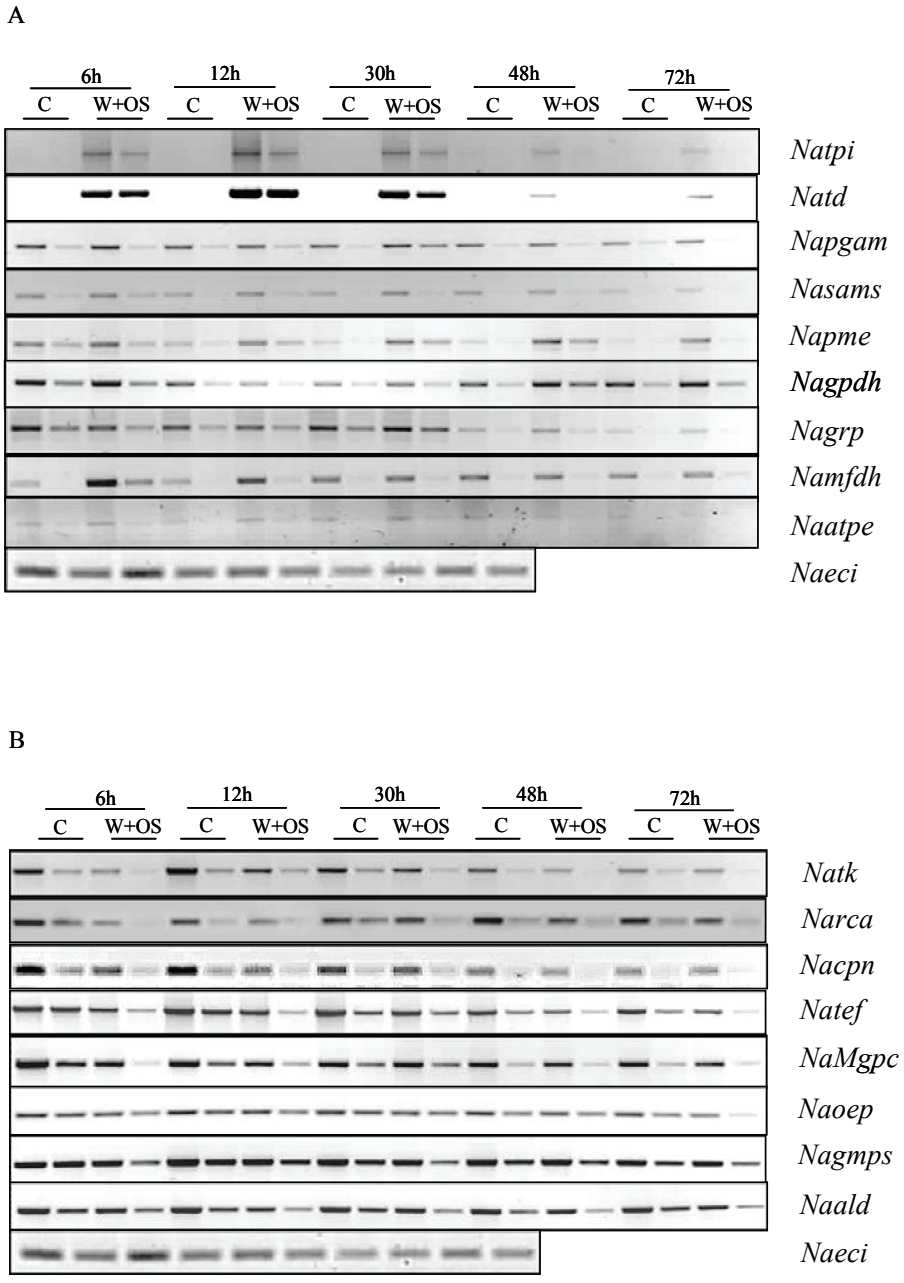


Figure 7. Gene expression analysis of candidate proteins exhibiting differential accumulation in control and OS-induced *N. attenuata* leaves at different times after elicitation. Quantitative RT-PCR analysis of candidate genes in control leaves (C) and OS-induced leaves (W 1 OS) that show up-regulation (A; nine genes) and down-regulation (B; eight genes) between 6 and 72 h is shown. PCR reactions were carried out with two concentrations of cDNA for all primers, in at least three replicates. A single concentration of cDNA was used for ECI primers to determine the equal amount of the cDNA. The gene names, accession numbers, primer sequences, sizes of amplified cDNA fragments, and respective peptide sequence maps on proteins are provided in SupplementalTable S1 and Supplemental Figure S5.

Silencing *rca* in *N. attenuata* results in reduced photosynthesis rate and biomass, responses consistent with herbivore-attacked plants

Massive changes in RCA protein levels after elicitation were found in the proteomic analysis. Transcriptional analysis of induced tissues indicated that *Narca* gene is down-regulated after herbivory, supporting our earlier large-scale transcriptional analysis (Hermsmeier et al., 2001; Schittko et al., 2001). To understand the functional consequences of this herbivore-elicited down-regulation of RCA proteins in *N. attenuata* plants, we silenced its expression by virus-induced gene silencing (VIGS) in *N. attenuata* plants. Transcript levels of *Narca* were >50% reduced in rca-VIGS plants as compared to empty vector (EV) transformed plants **(Fig. 8A)**. Proteomic analysis of these plants revealed a significant reduction of all 7 RCA protein spots in control and OS-induced leaves as compared to EV plants (**Fig. 8 Ea-Ed).** This analysis provided a strong validation of the proteomic analytical procedures used in this study. The lower accumulation of RCA proteins correlated with decreased photosynthetic rates and biomass, and increased nitrate levels. The net photosynthesis rates of *rca*-VIGS plants were 40-50% lower compared to EV plants **(Fig. 8B)**. The slope of a regression of photosynthetic rate against C_i provides an *in vivo* measure of RuPBCase activity (Manter and Kerrigan, 2004), and the slope of the A/Ci curve of EV plants is ~1.5 times greater than that of the PTV-*rca* plants, which clearly demonstrates that silencing of RCA reduced RuBPCase activity, which in turn decreased net photosynthesis. The biomass of *rca*-silenced plants was reduced by 48-50% (**Fig. 8C**). In contrast nitrate concentrations were significantly higher in *rca*-VIGS plants than in EV plants (**Fig. 8D**).

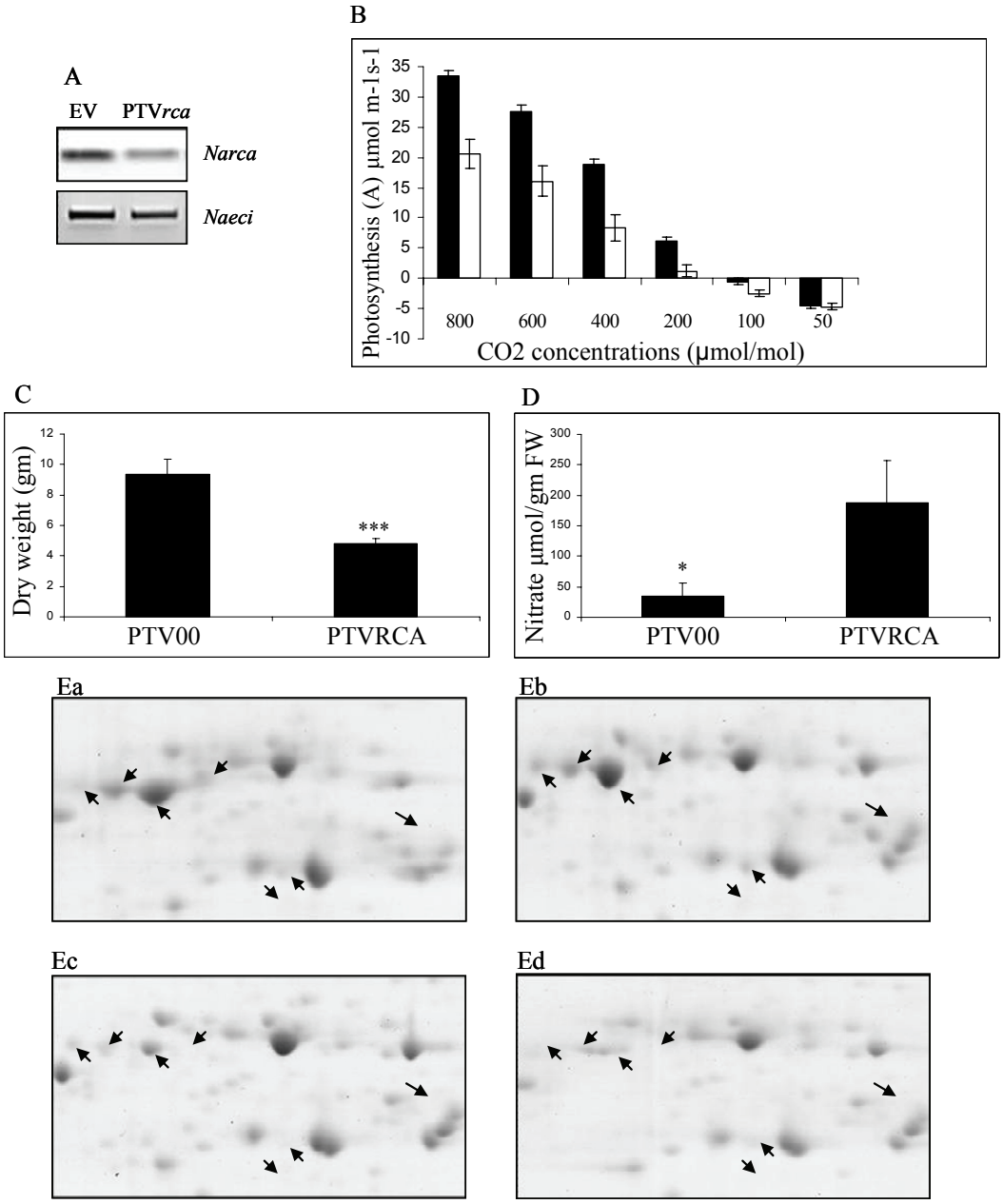


Figure 8: Effect of RCA reduction on photosynthesis, biomass, and nitrate content in *N. attenuata*. Three-week-old *N. attenuata* plants were inoculated with *Agrobacterium tumefaciens* harboring a TRV-based construct containing a 268 bp fragment of *Narca* or EV constructs for the VIGS experiments. **(A)** Gene silencing was confirmed at transcript and **(Ea- Ed)** proteomic levels. RCA spots are from *rca*-silenced plants compared with EV transformed plants, shown are EV control leaves **(Ea),** EV leaves treated with OS **(Eb),** *rca*-VIGS control leaves **(Ec),** and *rca*-VIGS leaves treated with OS **(Ed). (B)** Net photosynthetic rates and intercellular CO_2 concentrations of *rca*-VIGS plants (empty bars) were measured and compared to EV control plants (filled bars) under saturating light (~1400µM m^{-2} s^{-1}) intensities using Li-Cor 6400 Portable Photosynthesis System (LiCor Biosciences, Lincoln, USA). **(C)** Seven-week-old plants were harvested and dried in the oven at 60°C for 48 h for dry mass measurements. **(D)** The nitrate content of these plants was measured spectrophotometrically.

Discussion

We conducted a proteomic analysis of a natural plant-herbivore interaction for which previous research had characterized the host plant's transcriptional responses and the larval elicitors that activate them. From this analysis of 90 herbivore-elicited protein spots in over 84 2DE gels, we not only developed the techniques for extracting, separating, and analyzing complex protein mixtures reproducibly, we also characterized proteomic responses to elicitation and learned that the larval elicitors responsible for the transcriptional response can also account for a majority of the specific changes in the proteome. Previous transcriptional analyses of this particular interaction, as well as of other plant-herbivore interactions, can be broadly seen as representing a shift in metabolism from growth- to defense-related processes (Walling, 2000; Reymond et al., 2004; Bostock, 2005; Leitner et al., 2005; Schmidt et al., 2005; Thompson and Goggin, 2006). We organize the discussion into five sections: the change in proteins associated with 1) photosynthesis; 2) primary and 3) secondary metabolism as these proteins relate to supporting known herbivore-elicited defense responses; 4) the kinetics and elicitors of the transcripts for a subset of 17 elicited proteins, and 5) the signals from the feeding larvae responsible for changes in the proteome.

Changes in the photosynthesis-related proteome

We identified 7 different functional photosynthesis-related proteins (from the analysis of 20 protein spots), which accumulate differentially in herbivore-induced leaf tissues. We excluded RuBPCase from the analysis because its transcripts are known to degrade in response to herbivore and pathogen attack (Hermsmeier et al., 2001; Hahlbrock et al., 2003; Hui et al., 2003). Western-blot analysis of 2DE separated leaf proteins with two different RuBPCase antibodies revealed extensive fragmentation of this protein in response to chilling of rice plants (Yan et al., 2006). Given these stress-associated dynamics, it is not surprising that we found 7 RCA protein spots which responded to herbivore elicitation. Yan et al. (2006) identified 5 RCA spots in rice leaf proteins which responded to chilling. Since the RCA protein is involved in activating RuBPCase and maintaining its active state, the dynamics of these two very abundant proteins are clearly interdependent.

RCA belongs to the AAA^+ protein super family, whose members generally function as chaperonins, and uses the Sensor-2 domain to recognize its substrate (Vargas-Suarez et al., 2004; Kirch et al., 2005). Our analysis revealed substantial changes in RCA protein spots by herbivore elicitation, which suggests that the regulation of the RCA subunit content and its composition may contribute to optimizing plant performance during attack. How this occurs

requires further study. Chaperones assist in folding a wide range of structurally and functionally unrelated proteins, and it is possible that RCA interacts with proteins other than RuBPCase when plants are attacked. As is consistent with the existence of alternative functions, RCA is expressed in the nonphotosynthetic seeds of monocots and dicots which lack RuBPCase (Vargas-Suarez et al., 2004).

RCA modulates the activity of RuBPCase, a key regulatory enzyme of photosynthetic carbon assimilation, by facilitating the removal of sugar phosphates (ribulose bisphosphate) that prevent substrate binding and carbamylation of the protein's active site. The VIGS experiment demonstrated that reducing RCA protein and transcript levels as occurs in OS-elicited plants, reduced net photosynthetic rates of *N. attenuata* plants, which in turn was reflected in reduced biomass of these plants. The role of RCA in regulating the activity of RuBPCase (Portis, 1995), was clearly seen in the *in vivo* measures of RuBPCase activity, which were reduced in *rca* silenced lines. RCA-mediated reductions in carbon gain also inhibited the plant's ability to reduce and assimilate nitrate, the concentrations of which increased in *rca*-silenced plants. Numerous studies have shown that genetic or environmental manipulations that decrease photosynthesis also strongly inhibit nitrate assimilation (Matt et al., 2002). It has been shown that herbivore-induced reductions in photosynthetic rates are greater than the amount of canopy area removed by the herbivore (Zangerl et al., 2002) and these results point to herbivore-induced reductions in RCA protein as a potential explanation for the unexpectedly large decrease in photosynthetic rate in attacked leaves.

In addition to RCA, five other photosynthetic proteins were found to be down-regulated during all harvests after elicitation: three oxygen-evolving enhancer proteins and two oxygen-evolving complex proteins. In contrast, photosystem I chain II D2 precursors and S2 hydroxy acid oxidase proteins increased after OS elicitation. These results confirm our earlier transcription analysis of *N. attenuata-M. sexta* interaction, which revealed that most photosynthesis-related genes are down-regulated after herbivore attack (Halitschke et al., 2001; Hui et al., 2003).

The potential defensive role of the elicited proteins of primary metabolism

Of the 28 primary metabolism proteins that were differentially regulated, 8 are involved in amino acid metabolism and were up-regulated in response to OS-elicitation: TD, S-adenosylmethionine synthetase, O-acetylserine thiol lyase, spermidine synthase, glutamine synthetase, 3-dehydroquinate dehydratase, homocysteine-S-methyltransferase, and hydroxymethyltransferase. Chen et al. (2005) recently demonstrated a defensive role for

tomato TD, the enzyme that functions as an anti-nutritional protein in the first committed step in isoleucine biosynthesis by degrading threonine and presumably limiting the availability of this essential amino acid to the feeding insect. It is possible that the other up-regulated amino-acid-related proteins in *N. attenuata* leaves play a role in disturbing the amino acid metabolism of insects and thereby function as antinutritional defenses.

A protein spot identified as an Acyl CoA synthetase (ACS) had a type III response. Several *acs* genes reportededly played diverse roles in plant metabolism (Hayashi et al., 2002; Schnurr et al., 2004). A member of the *acs* gene family (*opcl1*) in Arabidopsis is reported to be responsive to wounding and is involved in jasmonic acid biosynthesis (Koo et al., 2006). Jasmonic acid is a well-characterized lipid-derived signal molecule that regulates defense-related processes in plants. The *acs* gene is also expressed in the epidermal layers of young and rapidly growing leaves of *Arabidopsis*, suggesting that the ACS enzyme may act to synthesize cutin or cuticular waxes (Hayashi et al., 2002; Schnurr et al., 2004). Cutin-reinforced leaves could decrease the palatability of leaves for insects. Another protein similar to glyceraldehyde-3-phosphate dehydrogenase (GADPH) protein exhibited differential accumulation in elicited leaves. Pathogen elicitation of maize cell suspension cultures elicits the accumulation levels of GAPDH (Chivasa et al., 2005) in Arabidopsis and is known to be regulated by redox (Hancock et al., 2005), and reversibly inhibited by nitric oxide (Lindermayr et al., 2005). These characteristics suggest that in addition to its catalytic role in glycolysis, GAPDH may be involved in reactive oxygen species signaling, which is important in plant-herbivore interactions.

We found proteins similar to transketolase (TK) and plastidic aldolase (ALD) to be differentially regulated by OS elicitation. High levels of accumulation of TK protein after OS elicitation might provide substrates for the synthesis of phenolic-based defenses. Proteomic analysis of germinating maize embryos infected by *Fusarium verticilliodes* showed the increased accumulation of ALD proteins (Campo et al., 2004). Gene expression of ALD is regulated by light and environmental factors, and thus might be influenced by photosynthetic activity and/or regulated by other photosynthesis-related genes (Yamada et al., 2000) as we also observed in *N attenuata*. Another protein similar to phosphoglycerate mutase (PGM) is up-regulated in OS-induced leaves. Expression of the *Arabidopsis* PGM is known to be regulated by hormones and glucose and induced by sedentary plant-parasitic nematodes (Mazarei et al., 2003; Bourgis et al., 2005). After elicitation, proteins with similarity to L6, L9 and L30 type ribosomal proteins increased, while proteins similar to the L4 and L6 type decreased. Ribosomal proteins contribute to the structures required for protein biosynthesis, as

do translation elongation factors, which catalyze the translocation of the two tRNAs and the mRNA after peptidyl transfer to the 80 S ribosome (Jorgensen et al., 2006). In rice, translation elongation factor genes are reported to be inducible in response to several environmental stresses and to abscisic acid treatment (Li et al., 1999). The changes observed in the *N. attenuata* translation elongation factor protein and several ribosomal proteins suggest a massive reorganization occurs in protein biosynthesis after herbivore damage to leaf tissues.

The potential defensive role of the elicited proteins of secondary metabolism

Seven out of eight proteins involved in the production of secondary metabolites showed increased accumulation levels at at least one time point after OS elicitation. Levels of a quinone oxidoreductase (QR)-like protein increased after OS elicitation. QRs are multi-subunit enzymes that are involved in the generation of free radical semiquinones. Recently, QRs genes have been shown to be up-regulated in the epidermis during powdery mildew infection to wheat (Greenshields et al., 2005). A protein spot similar to dehydroascorbate reductase (DHAR) showed a type I response after OS induction. DHAR functions in the regeneration of ascorbate, a potent antioxidant protecting plants against oxidative damage imposed by environmental stresses. It has been demonstrated in Arabidopsis that ascorbate deficiency can lead to the modification of defense pathways (Kiddle et al., 2003). Another protein similar to 3-dehydroquinate dehydratase, which was up-regulated, is known to be involved in the synthesis of lignin, chlorogenic acid, alkaloids, indole, other aromatic compounds, and tannins (Bischoff et al., 2001; Schmelz et al., 2006). The increased levels of this protein confirm results from our earlier studies, which show that several genes involved in the shikimate pathway are up-regulated in response to herbivory in *N. attenuata* (Baldwin, 2001). Importantly, a protein with similarity to cinnamyl-alcohol dehydrogenase, which is further downstream of the shikimate pathway, was also up-regulated. This enzyme catalyzes the reduction of phenylpropenyl aldehydes to monolignols, the last monomers before lignin synthesis (Kim et al., 2004). Lignification is an important process that facilitates tissue repair, increasing the toughness of leaves which in turn inhibits bacterial growth or can make feeding difficult for chewing insects (Chittoor et al., 1997).

A protein spot showing similarity to spermidine synthase is up-regulated from 6 to 72 h after OS elicitation. Spermidine synthase is involved in polyamine metabolism and thereby the production of secondary metabolite defense compounds (Franceschetti et al., 2004). This enzyme also catalyzes the first specific step in the biosynthesis of the tropane and nicotine alkaloids (Franceschetti et al., 2004; Stenzel et al., 2006). High levels of nicotine in *N.*

attenuata leaves in response to herbivory have been demonstrated in several studies (Baldwin, 2001; Steppuhn et al., 2004). Transgenic *N. attenuata* plants with reduced levels of constitutive or induced nicotine were preferred by insects in choice assays, and when these plants are grown in their native habitat, they are highly susceptible to several other insect pests (Steppuhn et al., 2004). Another protein similar to S-adenosyl-methionine synthase accumulates in elicited leaves and might be responsible for the supply of adenosylmethionine in ethylene biosynthesis (Urao et al., 2000). Increase in S-adenosyl-methionine synthase corresponds with the dramatic burst of ethylene and nicotine production that occurs when *M. sexta* larvae feed on *N. attenuata* leaves (Baldwin, 2001; Steppuhn et al., 2004). Here we were able to confirm these findings by demonstrating that S-adenosyl-methionine synthase and spermidine synthase proteins accumulate after herbivore attack.

Comparing changes in mRNA and protein accumulation

It is evident from several studies that proteomic analysis of a plant's response does not necessarily match the transcriptional analysis of the same response. In the present study also one of the major constraints is linking protein accumulation with mRNA accumulation pattern. Presence of several isoforms and/or subunits of certain proteins are the probable reasons for this discrepancy. This is especially important in cases where the candidate proteins belong to a multi-gene family. Therefore, a discrepancy in protein and transcript accumulation can not easily be ruled out. In the well-studied eukaryotic system of yeast, similar mRNA expression levels were accompanied by a wide range of protein abundance levels and *vice versa* (Gygi et al., 1999). The chilling of rice plants results in changes in the abundance of several mRNA transcripts, which are not reflected in changes in their corresponding proteins (Yan et al., 2006). We compared accumulation levels in leaves of 17 gene transcripts with those of their corresponding proteins at five time points after elicitation with different insect-derived signals. Six genes showed transcript patterns that correlated strongly with the patterns of protein abundance in the time series analysis. We used the *Natpi* gene for expression analysis, as it is known to respond to OS elicitation and insect damage at both transcript and protein levels (Zavala et al., 2004). For TPI, TD, glycine-rich RNA-binding protein, phosphoglycerate mutase, RCA, and glutamine synthase, the abundance of mRNA was a rough predictor of protein abundance, a relationship that has been shown for other proteins (Futcher et al., 1999). *Ald* (spot 19) showed an inconsistent pattern of protein accumulation, whereas at its transcript level we found a type II pattern of expression (always down). The mRNA and protein levels of the remaining ten proteins were differently regulated when compared in the time series analysis after OS elicitation. Comparison of protein and

mRNA accumulation after different elicitation treatments identified seven members, namely *tpi*, *td*, *pgm*, *sams*, *fdh*, *gpdh*, and *atpe*, which shared similar patterns. For the *cpn*, protein and mRNA levels were similarly regulated only after larval feeding but not by the other elicitation treatments. Pectin methyl esterase and *ald* were not regulated by the different elicitors at the protein level, but all treatments elicited strong increases at the transcript level.

Insect-derived signals and protein accumulation patterns in *N. attenuata* leaves

The patterns of differential protein accumulation as well as the identity of the elicited proteins provide insights into the nature of the larval elicitors that are involved in regulating plant proteomes. Previous research using a detailed microarray and secondary metabolite analysis demonstrated that the two most abundant FACs in *M. sexta* OS can account for all measured direct (TPI and nicotine) and indirect (*cis*-α-bergamotene) defenses, the endogenous jasmonic acid burst that elicits them, and 65-86% of the induced transcriptional changes elicited by attack on *N. attenuata* leaves (Halitschke et al., 2001, 2003; Roda et al., 2004). Here we extend the analysis to the level of the proteome and find treatment of wounds with four synthetic FACs can account for 19 of the 32 proteins elicited by W+OS treatment. Moreover, excluding FACs from OS by ion-exchange chromatography removes the regulation of 8 proteins, and treating FAC-free OS elicits only one additional protein, which was not elicited by treatment of the synthetic FACs. It is interesting to note that the response of certain proteins can not be attributed to the presence of FACs (**Table II, Fig. 5**). For example, RCA spots 20, 46, and 51 exhibit distinctly higher accumulation levels in leaves after treatment with OS and FAC-free OS compared to leaves after treatment with synthetic FACs, suggesting that unknown factors in OS modulate the elicitation activity of FACs.

FACs are plant defense elicitors which are synthesized in the larval mid-gut and elicit defense responses in the plant that appear to benefit the plant. However, other elicitors are produced by the plant during caterpillar attack and some of these may benefit the caterpillar. Recent work from our group has shown that treatment of wounds with OS and larval attack dramatically increases methanol (MeOH) emissions from attacked plants. This MeOH release is elicited not by the FACs in OS but, rather, by the high pH of OS (pH 9.3), and is associated with increases in transcripts and activity of leaf pectin methylesterase (PME) and decreases in the degree of pectin methylation (von Dahl et al., 2006). The OS elicitation of PME transcripts and activity is consistent with the hypothesis that methylated pectin can account for the MeOH emission (von Dahl et al., 2006). The results of this study confirm these conclusions and extend the analysis to the levels of PME protein which we found to increase

after wounding and application of OS at 30 and 72 h after elicitation (**Table I**). This MeOH emission results in decreased TPI levels, but how this works has not been discovered; the decreased TPIs enhance larval performance (von Dahl et al., 2006) and it appears that the feeding larvae generates a beneficial signal by exploiting an indispensable feature of cell wall pectin chemistry essential for plant growth.

In conclusion, FACs play a major role in organizing not only the transcriptional responses but also the proteomic responses. This dual role is not simply a result of an overlap of the transcriptional and proteomic responses to FACs, because the responses of the transcriptome and proteome are clearly very different.

Materials and Methods

Plant material and preparation of elicitors

A seventeenth-generation inbred line of *N. attenuata* (ecotype Utah), originally collected from a natural population in Washington County, Utah, USA, was used for all experiments. Seed germination and plant growth were conducted as described earlier (Halitschke et al., 2003). All elicitation experiments were conducted with plants in the rosette stage of growth. *M. sexta* eggs were collected from Lytle Preserve in Utah and bred in our laboratory. To elicit plants, freshly hatched first-instar caterpillars reared on *N. attenuata* wild-type plants were released on leaves growing at particular positions. OS were collected from fourth- and fifth-instar larvae. Collected OS were stored at -20°C under argon and diluted 1:1 (v/v) with sterile water prior to use (Halitschke et al., 2003).

The FAC-solution was prepared as described earlier (Halitschke et al., 2001). To obtain OS without FACs (OS-FAC-free), OS were separated on ion-exchange Ultrafree-MC columns (Millipore, Billerica, MA, USA) that were filled with 300 mg basic anion-exchange Amberlite IRA-400 (Sigma, Steinheim, Germany). The removal of FACs from OS was verified by atmospheric pressure chemical ionization-HPLC-MS analysis using a Phenomenex C18(2) Luna, particle size 5 µm, 250 x 4.6 mm HPLC-separation column (Phenomenex, Torrance, CA, US) as described earlier (Halitschke et al., 2001).

Elicitation experiments

Source leaves +1, +2, and +3 of rosette-stage plants of *N. attenuata* (**Fig. 1**) were punctured parallel to the central leaf vein with a fabric pattern wheel six times at 30-min intervals. Induction treatments were started between 9 and 10 am and performed every 30 min for 3 h. Around 20 µL of 1:1 water-diluted OS or OS-FAC-free or FACs (in 0.0025% Triton X-100) or sterile water or 0.0025% Triton X-100 were applied to the punctured leaves (Halitschke et al., 2001, 2003). Local leaves were harvested for protein and RNA extraction after 6, 12, 30, 48, and 72 h. Systemic leaf samples (leaves -1, -2, and -3) were collected 30 h after the initial induction treatment (**Fig. 1**). For the caterpillar-feeding treatments, 15 *M. sexta* neonate larvae (5 larvae per leaf) were released on +1, +2, and +3 leaves and their feeding patterns were monitored frequently to ensure that only the designated leaves were attacked. Damaged leaves were harvested after 30 and 48 h. All harvested tissues were frozen in liquid nitrogen and stored at -80^{o}C prior to protein extraction. The complete experiment was repeated at least three times each with a minimum of three biological replicates.

Protein extraction, separation by 2DE, and analysis of protein spots

Total protein was extracted from elicited and control leaves with a modified phenolic extraction procedure (Schuster and Davies, 1983; Saravanan and Rose, 2004). For nuclear protein extraction, the ReadyPrep Protein Extraction Kit was used according to manufacturer's instructions (Biorad, Munich, Germany). Protein quantification was carried out with a 2DE Quant kit according to the manufacturer's instructions (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). For first-dimension separation, 24 cm IPG strips pH3-10 NL (Biorad, Munich, Germany) were rehydrated with 550 µg (410 µL rehydration buffer) of protein for 11-12 h at room temperature. The proteins were focused on a Protean IEF Cell (Biorad, Munich, Germany) at 20°C, rapid voltage ramping, 63000 volt h, and 50 µA current per IPG strip. Prior to second-dimension separation, the IPG strips were equilibrated with a DTT buffer (3.6 g urea, 5 mg dithiothreitol, 1 mL glycerol, 4.4 mL 10% SDS, and 1 mL 0.6 M Tris-HCl buffer pH 6.8 in 10 mL) followed by an iodoacetamide buffer (similar to DTT buffer, with 0.25 g iodoacetamide instead of DTT), 20 min each at room temperature on a shaker. SDS-PAGE for second-dimension separation was performed with 12% gels and the Ettan Daltsix electrophoresis unit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) at 25°C and 17 W/gel. The gels were stained with Bio-safe Coomassie G-250 (Biorad, Munich, Germany) according to the manufacturer's instructions. Gel images were created using a GS-

800 calibrated densitometer (Biorad, Munich, Germany). Spot detection, indexing, matching, normalization, and quantitation were done using the PDQuest v7.3.1 software (Biorad, Munich, Germany). Protein spots of interest were excised and used for tryptic protein digestion. The p-values were calculated with at least three 2DE images of the corresponding biological replicates. The ratios of the relative spot quantities of the induced 2DE images between changing spots and an unchanged reference spot from the same 2DE image were calculated and compared with a two-tailed and paired *t*-test against the relative spot quantity ratios from the control images for the respective treatments (**Table II**).

Protein digestion for MS analysis

Proteins of interest were processed on 96-well microtiter plates with an Ettan TA Digester running the Digester v.1.10 software (both GE Healthcare Bio-Sciences AB, Uppsala, Sweden) using the following protocol. The excised gel plugs were washed 4 times with 70 µL of acetonitrile/50 mM ammonium bicarbonate for 20 min each. The second wash was performed twice with 70 µL 70% acetonitrile for 20 min and the gel plugs were air dried for 1 h. Trypsin digestion was carried out overnight with 50 ng trypsin (Porcine trypsin, Promega) in 15 µL 50 mM ammonium bicarbonate at 37°C. The 15 µL solution from the preceding step was mixed with 25 µL extraction buffer (50% acetonitrile and 0.1% trifluoroacetic acid), incubated for 20 min and transferred to a 96-well plate. The gel plug was then incubated with 40 µL of extraction buffer for 20 min and then transferred to the plate. The 60 µL solution containing the peptide mixture was then vacuum-dried for 1h.

MALDI-TOF and LC-MS/MS analysis of protein spots

Dry peptides were dissolved in 15 µL aqueous 0.1% triflouroacetic acid. One µL aliquot was mixed with 1 µL α-cyano-4-hydroxy cinnamic acid (alpha matrix, 10 mg/mL in ethanol/acetonitrile 1:1 v/v), and 1 µL of this mixture was transferred onto a metal 96-spot MALDI target plate for co-crystallization. A MALDImicro MX mass spectrometer (Waters, Milford, MA, USA) was used in reflectron mode to analyze the tryptic peptides. A strong electrical field was created to accelerate the sample ions into the flight tube toward the detector (5 kV on the sample table, -12 kV on the extraction grid, pulse voltage of 1.95 kV, and 2.35 kV detector voltages). A nitrogen laser (337 nm, 5 Hz) was used for ionization, with energies of approximately 50 µJ per pulse. MassLynx v4.0 software served for data acquisition (Waters, Milford, MA, USA). Each spectrum was combined from 10 laser pulses.

Human Glu-Fibrinopeptide B (1570.6774 Da) served as an external lock-mass reference. Bovine serum albumin tryptic digest was used to calibrate the mass spectrometer (MPrep, Waters, Milford, MA, USA). The MALDI-TOF spectra searches were performed in the PLGS v.2.1.5 software (Waters, Milford, MA, USA) using the Green Plant v1.0 database of the NCBI. The search parameters were: peptide tolerance of 80 ppm, one missed cleavage, carbamidomethyl modification of cysteins and possible methionine oxidation. An estimated calibration error of 0.05 Da and a minimum of four peptide matches were the criteria for obtaining positive database hits. The MALDI-TOF peptide signal intensities were used to estimate the injection amount in the subsequent peptide sequence analysis.

The peptides, redissolved in aqueous 0.1% trifluoroacetic acid, were subsequently used for nanoLC-MS/MS analysis. The peptides were separated on a CapLC XE nanoLC system (Waters, Milford, MA, USA). A mobile phase flow of 0.1% aqueous formic acid (20 µL/min for 5 min) was used to concentrate and desalt the samples on a 5 x 0.35 mm Symmetry-300 C18 precolumn with 5 µm particle size. The samples were eluted and separated on a 150 mm x 75 µm NanoEase Atlantis C18 column, particle size 3 µm using an increasing acetonitrile gradient (in 0.1% aqueous formic acid). Phases A (5% MeCN in 0.1% formic acid) and B (95% MeCN in 0.1% formic acid) were linearly mixed using a gradient program set to 5% phase B in A for 5 min, increased to 40% B in 25 min, and to 60% A in 10 min, and finally increased to 95% B for 4 min. The peptides were directly transferred to the NanoElectroSpray source of a Q-TOF Ultima tandem mass spectrometer through a Teflon capillary union and a metal coated nanoelectrospray tip (Picotip, 50 x 0.36 mm, 10 µm I.D) (Waters, Milford, MA, USA). The source temperature was set to 40°C, cone gas flow 50 L/h, and the nanoelectrospray voltage was 1.6 kV. The TOF analyzer was used in reflectron mode. The MS/MS spectra were collected at 0.9 s intervals in the range of 50-1700 m/z. A mixture of 100 fmol/µL human Glu-Fibrinopeptide B and 80 fmol/µL reserpine in 0.1% formic acid/acetonitrile (1:1 v/v) was infused at a flow rate of 0.9 µL/min through the reference NanoLockSpray source every fifth scan to compensate for mass shifts in the MS and MS/MS fragmentation mode due to temperature fluctuations. The data were collected by MassLynx v4.0 software. ProteinLynx Global Server Browser v2.1.5 (RC7) software (both from Waters, Milford, MA, USA) was used for further data processing (deconvolution, baseline subtraction, smoothing), *de novo* sequence identification, and database searches. The MS/MS data were searched against the NCBI Green Plant v1.0 database with the following parameters: peptide tolerance of 20 ppm, fragment tolerance of 0.05 Da, estimated calibration error of 0.005 Da, 1 missed cleavage, carbamidomethylation of cysteins, and possible oxidations of methionine.

The amino acid sequences of peptides which did not provide conclusive results from the database searches were searched using MS-BLAST internet (http://www.dove.embl-heidelberg.de) and an in house-based search engine. Consolidated database (sp_nrdb) containing all non-redundant protein databases: SwissProt, SwissProtNew, SptremblNew, Sptrembl, PAM30MS matrix, and default setting were used for searches (Shevchenko and Shevchenko, 2001).

RT-PCR analysis

The total RNA was extracted from local leaves as described in the TRI reagent protocol (Sigma, Taufkirchen, Germany). From all the samples 1 µg of total RNA was converted to cDNA using a Superscript first strand synthesis system for RT PCR according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

Primers were designed from the peptide sequences obtained after LC-MS/MS analysis. Respective sites of the peptides were mapped on nucleotide regions for the respective database entries. Closely related species of *N. attenuata* were used for primer design when the sequence information of particular genes was unavailable. The primer design scheme is provided in **Supplemental Table I**. The location of the peptides within the protein sequence is provided in **Supplemental Figure 5.**

Two concentrations of cDNA (*viz.* 1x, 5x or 50x, 250x dilutions of original cDNA derived from 1 µg of RNA) derived from control and induced leaves were used as a template to amplify the respective cDNA fragment with the above primer combinations. PCR conditions were optimized for each primer set. PCR was carried out after denaturing cDNAs at 94^{o}C for 5 min and then 30 cycles of 94^{o}C for 60 s, annealing temperature (see **Supplemental Table I**) for 30 s, and extension at 72^{o}C for 60 s. The final extension step in PCR was at 72^{o}C for 10 min. The amplified cDNA fragments were purified from agarose gel using GFX gel elution kit (Amharsham, Germany) and cloned into PGMT-Easy vector (Promega, Madison, WI, USA). Cloned fragments were sequenced and confirmed with the NCBI database and novel sequences were deposited in the database.

***Narca* gene silencing and analysis**

The previously reported *N. attenuata* 268 nucleotide *rca* sequence (BU 494545) was used to make the TRV-based construct for virus-induced gene silencing (VIGS) as described earlier (Saedler and Baldwin, 2004). Three-week-old *N. attenuata* plants were inoculated with *Agrobacterium tumefaciens*, transformed with PTV-*rca* or PTV-*phytoene desaturase* (*pds)* or empty vector (EV, PTV00) constructs (Saedler and Baldwin, 2004; Rayapuram and Baldwin,

2006). Plants were grown in growth chambers at 22°C for two to three weeks until leaves of *pds*-transformed plants showed bleaching, a symptom of VIGS (Saedler and Baldwin, 2004). Total RNA from EV and *rca*-VIGS plants was extracted and analyzed for *rca* expression using RT-PCR. Induction to +2 leaves with W+OS was carried out with five replicate plants per treatment as described in earlier sections. Protein extraction and 2DE analyses of the leaf were carried out and compared with EV transformed plants.

Five-week-old plants were used for photosynthesis and nitrate measurements. Net photosynthetic rates and intercellular CO_2 concentrations were measured on empty vector and *Narca* VIGS-silenced plants under saturating light (~1400μM m^{-2} s^{-1}) using Li-Cor 6400 Portable Photosynthesis System (LiCor Biosciences, Lincoln, USA). Net photosynthesis was measured in at least five replicate plants each at six different CO_2 concentrations, namely, 800, 600, 400, 200, 100, and 50 μmol/mol. Nitrate was measured spectrophotometrically according to the protocol described by Cataldo et al. (1975). Seven-week-old plants were harvested and dried in the oven at 60°C for 48 h and dry weights of the plants were measured.

Accession numbers

New sequences of putative partial genes of *N. attenuata* cloned and characterized in the present study were deposited in the public database NCBI. Accession numbers allotted to these genes are as follows: *N. attenuata* (*Na*) ATPase, *Naatpe* (DQ682456); chaperonin, *Nacpn* (DQ682457); glutamine synthase, *Nagmps* (DQ682458); glyceraldehydes-3-phosphate dehydrogenase, *Nagpdh* (DQ682459); glycine-rich RNA binding protein, *Nagrp* (DQ682460); Mg protoporphyrin IX chelatase, *NaMgpc* (DQ682461); oxygen-evolving protein, *Naoep* (DQ682462); RuBPCase activase, *Narca* (DQ682463); S-adenosylmethionine synthetase, *Nasmas* (DQ682464); translation elongation factor, *Natef* (DQ682465); phosphoglycerate mutase, *Napgam* (DQ682466); aldolase, *Naald* (DQ682467); transketolase, *Natk* (DQ682468); mitochondrial formate dehydrogenase, *Nafdh* (DQ885565) and pectin methyl esterase, *Napme* (DQ885566).

ACKNOWLEDGMENTS

APG acknowledges the Alexander von Humboldt Foundation, Bonn, Germany, for a research fellowship. We thank Klaus Gase, Thomas Hahn, and Albrecht Berg for DNA sequencing, providing chemically synthesized FACs and HPLC analysis, Tamara Krügel and Andreas Weber for growing the plants, and Emily Wheeler for editorial assistance. We also

thank Markus Hartl for critically reading the MS and Nan Qu for assistance in the preparation of the nuclear extracts and for helpful discussions.

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3.2 Manuscript II

Classification: Biological sciences, chemical ecology

Silencing 7 herbivory-regulated proteins in *Nicotiana attenuata* to understand their function in plant-herbivore interactions

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Proceedings of the National Academy of Sciences of the United States of America: In review

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Key words: Herbivore-responsive proteins, *Manduca sexta*, *Nicotiana attenuata*, plant defense, plant growth, virus-induced gene silencing

Manuscript information: 18 text pages, 2 tables, 3 figures, 4 supplemental tables, and 5 supplemental figures.

Word count: words in abstract 235; total characters with spaces 47,297.

Summary

Unbiased "omic" tools reveal that a plant's primary metabolism is reconfigured when herbivores attack. However, unbiased means of evaluating the function of this reconfiguration for the plant-herbivore interaction are lacking. The production of secondary metabolites can be genetically silenced for *in planta* tests of defensive function without dramatically altering growth. This is not possible with primary metabolites, which may also function defensively. We test this hypothesis by silencing the expression of genes coding for 7 herbivore-induced proteins not known to play obvious defensive roles in the *Nicotiana attenuata-Manduca sexta* interaction and analyzed the performance of plants and insects traits known to mediate resistance and performance. Silencing genes of up-regulated proteins in *N. attenuata* tended to benefit larval performance, while silencing genes of down-regulated proteins tended to decrease it: these results are consistent with expectations that herbivory-induced regulation reflects the proteins' function in attacked plants. Larval midgut protein analysis by 2D-gel electrophoresis revealed a dynamic proteomic response to diets of plants silenced in herbivory-regulated proteins. Surprisingly, plant trypsin proteinase inhibitors (TPIs) and larval midgut protease activity were not associated with larval performance and neither was the variation in established defense metabolites (nicotine), suggesting greater importance of other metabolites, such as diterpene glycosides. We conclude that by silencing the genes of proteins differentially regulated during a plant-herbivore interaction and querying each player in the interaction can reveal novel functions of genes at an organismic level of analysis.

Introduction

Plants use numerous dynamic defense strategies against biotic attackers (1, 2). For example, plants dramatically increase the production of secondary metabolites. These secondary metabolites function as defenses by directly reducing the consumption, growth, and reproduction of insects because they inhibit digestive enzymes or are toxic to some aspect of insect physiology (3). However, unbiased "omic" approaches, which characterize transcriptomic, metabolomic, and proteomic changes in herbivore-attacked plants, have laid to rest the notion that metabolism can be neatly parsed into "secondary" components, used to meet environmental challenges, and "primary" components, used to support growth. Many different plant-herbivore interactions have been analyzed with microarrays and genes involved in many aspects of primary metabolism have been found to be regulated (for example, (4-8). This reconfiguration of primary metabolism may reflect changes in plant's growth processes required recover from herbivore attack, but the changes in primary metabolites function may either directly function as defenses or indirectly by influencing an herbivore's ability to detoxify secondary metabolites (9-11). This hypothesis can only be tested at the level of the organisms engaged in the interaction. With the development of transformation systems and the identification of genes that control secondary metabolite biosynthesis, it is now possible to rigorously test the defensive function of a secondary metabolite by engineering metabolite-deficient plants that otherwise grow normally and examining their performance in native environments (12-15). Testing the potential defensive function of an aspect of primary metabolism is a much greater challenge.

The defensive function of any aspect of primary metabolism is usually discovered by how it is regulated after herbivore attack, as the example of threonine deaminase (TD) illustrates. TD plays an essential role in the biosynthesis of the essential amino acid, isoleucine (Ile), but surprisingly the levels of its transcripts are dramatically increased in *Nicotiana attenuata* leaves in response to herbivore attack and are increased when mechanical wounds are treated with herbivore-specific elicitors. Silencing TD expression in plants without affecting growth increased herbivore performance, suggesting that TD plays a role in herbivore defense. Detailed analysis of plant defenses and the signals that elicit them revealed that TD supplied the Ile required to synthesize JA-Ile at the site of herbivore attack (16). Subsequently, JA-Ile has been discovered to be one of the most active jasmonates in eliciting herbivore resistance responses (17, 18). Additional proteomic analyses demonstrated that TD also functions as a post-ingestive defensive by catalytically degrading threonine, an essential

amino acid (19). This example illustrates the value of unbiased analyses of gene function at the organismic level; here we present an unbiased functional analysis for 7 herbivory-regulated proteins.

We conducted an organismic-level analysis of 7 differentially regulated proteins in the *M. sexta-N. attenuata* interaction. We used a virus-induced gene silencing (VIGS) system optimized for *N. attenuata* (20) to reduce the accumulation of the transcripts of the regulated proteins and examined the progress of the plant-insect interaction in currencies which reflect both sides of the interaction. From the plant's side, we analyzed the effect of gene silencing on plant growth, net photosynthetic rate, and the whole-plant accumulation of plant's secondary metabolites, namely nicotine, chlorogenic acid (CGA), rutin, diterpene glycoside (DTG), and trypsin proteinase inhibitors (TPIs). From the herbivore's side, we measured the mass gain of *M. sexta* larvae. To understand the differences in larval performance on the variously silenced lines, we measured the concentration of secondary metabolites and protease inhibitors in the attacked leaves. To learn more about how *M. sexta* larvae adjust their metabolism to diets of varying quality, we measured protease activity and conducted a proteomic analysis of midgut proteins of larvae that fed on the silenced lines.

To be selected, genes had to (i) have well-established kinetics of transcript and protein accumulation in the *N. attenuata-M. sexta* interaction, (ii) have no established association with defense in the *N. attenuata- M. sexta* interaction, and (iii) be involved in different processes *in planta*. Four up-regulated proteins – namely, vacuolar ATPase (ATPE), which is involved in ion and metabolite transport and cell enlargement (21); cinnamyl alcohol dehydrogenase (CAD), which is involved in the production of lignin monomers; chaperonin (CPN), which is involved in the association of RuBPCase subunits (22); and transketolase (TK), which is involved in Calvin cycle (23) – and three down-regulated proteins – namely, glyceraldehyde-3-phosphate dehydrogenase (GPDH), which is involved in glucose metabolism; glutamine synthase (GMPS), which is involved in the photorespiratory carbon cycle, where it re-assimilates ammonia (24); and oxygen-evolving protein (OEP), which is involved in the light reaction of photosynthesis -- were used in the gene-silencing experiments.

Results

Virus-induced gene silencing (VIGS) of 7 herbivore-elicited proteins

Twelve days after inoculation with the appropriate construct, the transcript levels of *Nacad*, *Nagmps*, *Naoep*, and *Natk* were reduced by more than 50%, and levels of *Naatpe, Nacpn,* and *Nagpdh* were reduced by more than 90% compared to the levels in empty vector (EV) inoculated plants (**Fig. S1**). The transcript levels of *Naatpe* are very low in EV-transformed plants and could not be detected in *Naatpe*-silenced plants. Silencing of some genes influenced plant growth and morphology (**Fig. 1A; S2**).

Photosynthetic capacity of silenced plants

Under saturating light conditions (~1200μmol PAR m^{-2} s^{-1}) and different CO_2 concentrations (200, 400, and 600 μmol mol^{-1}) pTV*atpe*, pTV*cpn*, pTV*gmps,* and pTV*tk* plants had only 20-50% of the photosynthetic capacity of EV plants, whereas photosynthetic rates of other lines were not significantly different from those of EV plants (**Fig. S3**). The plants were categorized by their photosynthetic performance, as unchanged or decreased. The slope of the A/Ci curve (an *in vivo* measure of RuBPCase activity) of EV plants was 2-5 times greater than that of the *atpe*-, *cpn*-, *gmps-, oep*-, and *tk*- silenced plants (25) **(Table S2)**.

Biomass and RuBPCase levels in silenced plants

Biomass of pTV*atpe*, pTV*cpn*, pTV*gmps,* and pTV*tk* plants was reduced by 50-90%; in contrast, pTV*cad* plants attained masses significantly larger (by 22%) than those of EV plants (**Fig. 1A**). The biomasses of the other lines did not differ from those of EV plants. RuBPCase levels, the most abundant protein (26), was significantly reduced in pTV*atpe*, pTV*gmps*, and pTV*oep* plants compared to EV-transformed plants. RuBPCase levels in the other silenced plants did not differ significantly from those in EV plants **(Figs. 1C)**. Moreover, RuBPCase levels were not significantly ($F_{1,7}$= 4.612, P=0.075) correlated with the A/Ci slopes, the *in vivo* measure of RuBPCase activity (Table S2), which suggests that substantial pools of inactivated RuBPCase had accumulated in some of the silenced lines.

Secondary metabolites

Levels of control and OS-elicited secondary metabolites were measured on a concentration basis (per unit leaf fresh mass) and also on a whole-plant basis (mass/plant).

Statistical analyses are presented in Table S3. Concentrations of CGA were higher in pTV*atpe,* pTV*cad* and pTV*cpn* plants than in EV plants. In contrast, *Natk*-silenced plants had lower CGA concentrations. Rutin concentrations in pTV*gmps* and pTV*tk* plants were significantly lower than those in EV-transformed plants (**Fig. 2D; Table S3**). Nicotine concentrations of pTV*atpe,* pTV*gmps,* pTV*gpdh,* and pTV*oep* plants were higher than those in EV-transformed plants (**Fig. 2E; Table S3**). DTG concentrations of pTV*cpn*, pTV*tk*, and pTV*gmps* plants were significantly lower compared to those in EV plants. OS-elicited DTG concentrations of pTV*tk* plants were significantly lower than in EV plants but in pTV*atpe* plants, levels were significantly higher (**Fig. 2F**). Compared to EV plants, *atpe*- and *tk*-silenced plants had higher constitutive levels of TPI activity; in contrast, constitutive and induced levels in pTV*gmps* plants were significantly lower than those in EV plants. (**Fig. 2G; Table S3).**

Similar patterns were found when the secondary metabolites were expressed on a whole-plant basis, with the exception of *Naatpe*-silenced plants, in which the patterns were reversed when expressed as whole-plant pools. *Naatpe*-silenced plants had significantly lower pools of CGA, rutin, nicotine, DTG, and TPIs compared to EV-transformed plants **(Figs. S3)**.

M. sexta larvae performance and midgut protease activity

Larvae that fed on *Nacpn*- and *Natk*-silenced plants gained significantly more mass than those that fed on EV plants. In contrast, larvae that fed on *Nagmps*-silenced plants gained significantly less mass, while larvae that fed on the other lines did not differ from those that fed EV plants. All larvae always had a surplus of leaf material to feed on and the differences in larval growth are not due to differences in the availability of food. To understand the differences in larval performance, VIGS lines were categorized according to their influence on larval mass gain, namely increased, not changed, and decreased in comparison to EV plants **(Fig. 2A; Table S3)**.

Protease activity in the guts of *M. sexta* that fed on pTV*gpdh* plants was higher than in the guts of EV-fed larvae, while protease activity in larvae that fed on pTV*gmps* and pTV*tk* plants was significantly lower. Larvae that fed on the remaining silenced lines had levels of protease activity similar to those that fed on EV plants (**Fig. 2H; Table S3**).

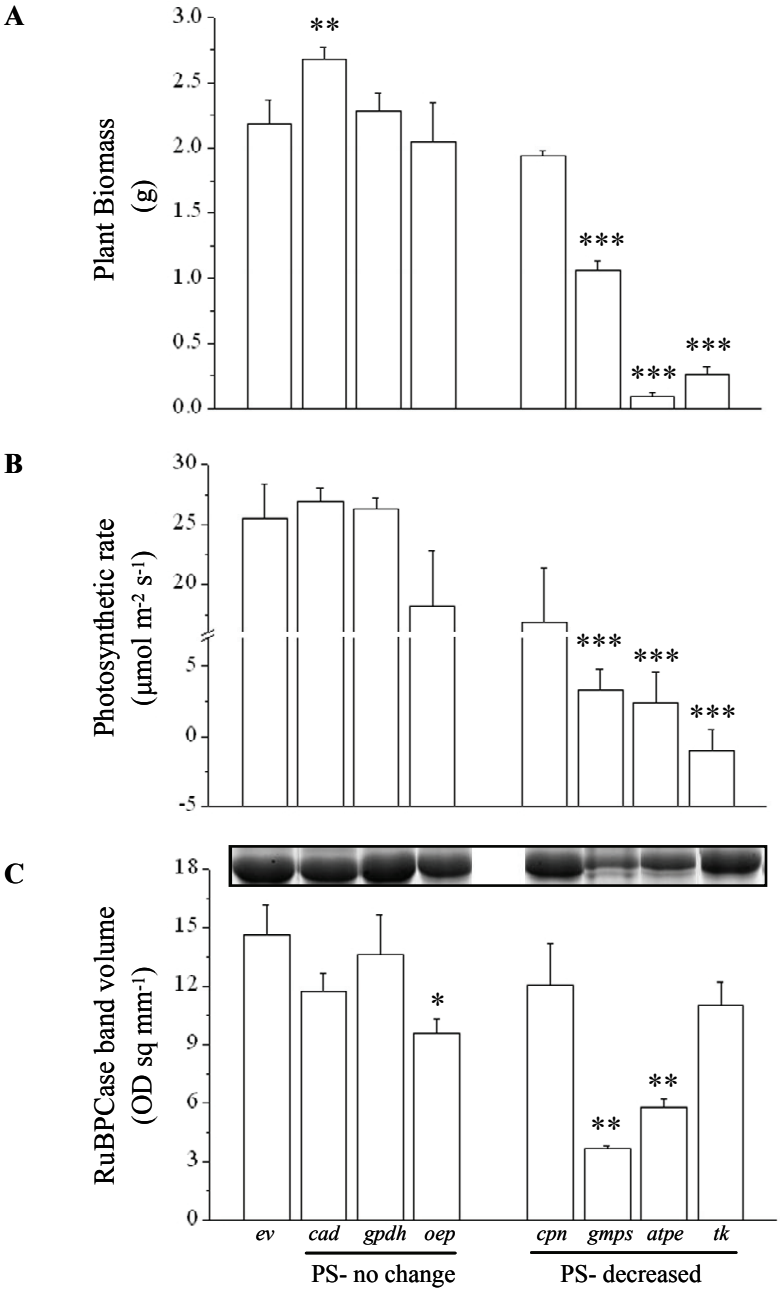

Fig. 1: Silencing candidate proteins decreases biomass in pTV*gmps*, pTV*atpe*, and pTV*tk* plants and increases biomass in pTV*cad* plants; variance in plant biomass was positively associated with photosynthetic capacity but not with RuBPCase levels. Mean (±SE) of 5 replicate plants of each VIGS line for **A**) biomass 30 days after inoculation, **B**) CO_2 assimilation (1200 µmol PAR m^{-2} s^{-1} and at 600 µmol CO_2 mol^{-1}), **C)** leaf RuBPCase concentrations as estimated by RuBPCase band volume (**inset**) from 1-D PAGE of 3 technical replicates. VIGS lines are categorized according to their photosynthetic performance (no change and decreased: see Fig. S3). Asterisks indicate significant differences at $P < 0.05$ (*), $P < 0.005$ (**), and $P < 0.0005$ (***).

Association of plant performance with photosynthetic capacity and whole-plant secondary metabolite pools

The production of defense-related metabolites is thought to interfere with plant growth and may even reduce a plant's fitness (14). To examine these relationships, the associations among plant biomass, photosynthetic capacity, levels of RuBPCase and defense metabolites were determined by multiple regression analysis. The analysis was performed in two groups. In the first, mean plant biomass was regressed against mean values of photosynthetic rate at high CO_2 concentrations (600μmol mol^{-1}) and RuBPCase contents; in the second, mean plant biomass was regressed against mean uninduced pool values of nicotine, CGA, rutin, DTG, and TPI for all genotypes. A significant positive relationship between photosynthetic capacity and plant biomass was found, but levels of RuBPCase, CGA, rutin, nicotine, DTG, and TPI were not significantly associated with plant biomass (**Table 1**; **Figs. 1, S4**).

Association of larval performance with RuBPCase and secondary metabolite levels, and gut protease activity

We used multiple regression analysis to understand the relationships among larval biomass, the levels of RuBPCase and the different secondary metabolites (**Table 2; Fig. 2**). The analysis was performed in two groups. In the first, mean larval mass was regressed against the mean photosynthetic rate at high CO_2 concentrations (600μmol mol^{-1}) and RuBPCase contents; in the second, mean larval mass was regressed against the mean OS-elicited concentrations of nicotine, CGA, rutin, DTG, TPI, and gut protease for all genotypes. The analysis revealed that rutin and TPI concentrations were positively associated with larval mass and negatively associated with DTG levels. However, photosynthetic capacity, levels of RuBPCase, CGA, nicotine, and gut protease were not significantly associated with larval mass. Therefore, the differences in larval performance cannot be attributed to the indirect effects of gene silencing on the production of secondary metabolites that are known to be defensive, namely nicotine and TPIs. The ingestion of plant TPIs was also not significantly associated with midgut protease activity (**Table 2; Fig. 2**).

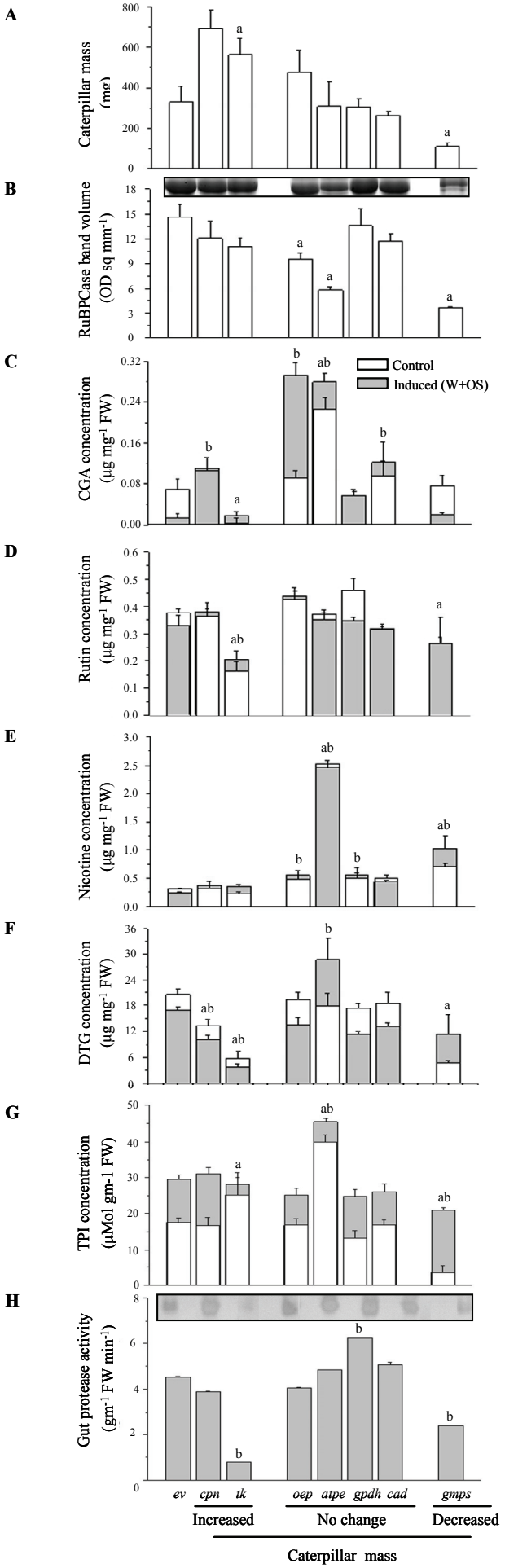

Fig. 2: *M. sexta* larval mass after 12 days of feeding on the different VIGS lines and their association with the concentrations of leaf secondary metabolite levels and midgut protease levels. **A)** Larval mass gain (mean± SE) of neonates after 12 days of feeding on the different VIGS plants. **B)** 1-D PAGE of a large subunit of RuBPCase in plants silenced in *Naatpe, Nacad, Nacpn, Nagpdh, Nagmps, Naoep,* and *Natk* plants compared to EV plants. The quantification revealed a decrease in the amount of RuBPCase in *Naatpe-*, *Nagmps-*, and *Naoep-*silenced plants compared to EV plants. 1-D PAGE image of silenced plants **(Inset 1).** Values are means (± SE) of three technical replicates. The levels of **C)** CGA, **D)** rutin, **E)** nicotine, **F)** DTG and **G)** TPI activity in leaves of all VIGS lines before and 4 days after W+OS elicitation. *M. sexta* midguts were dissected and analyzed for **H)** protease activity in larval gut. In-gel midgut protease activity assay of *M. sexta* larvae that fed on VIGS-silenced plants (**Inset 2)**. VIGS lines are categorized according to their effect on the mass (increased, no change, and decreased) of larvae that fed on them. Control and induced data sets are overlaid in order of size; bars labeled with a (control), b (induced), and ab (control and induced) to indicate significant differences compared to EV plants.

Table 1: The influence of silencing herbivory-regulated proteins on plant performance. The associations among plant biomass, photosynthetic capacity, levels of RuBPCase and secondary metabolites were determined by multiple regression analysis of mean values across all VIGS lines. The analysis was performed in two groups. In group 1, mean plant biomass was regressed against mean values of photosynthetic rate (600µmol CO_2 mol^{-1}) and RuBPCase contents; in group 2, mean plant biomass was regressed against mean uninduced pool values of secondary metabolites across all VIGS lines. All mean values were of five biological replicates.

Independent variables	Co-efficient (± SE)	t-Value	P-Value
Group 1 (R^2 = .926; adjusted R^2 = .897; F = 31.342; n = 8)			
RuBPCase level	-.014 (± .043)	-.327	.76
Photosynthetic rate	.08 (± .013)	6.05	**.0018**
Group 2 (R^2 = .984; adjusted R^2 = .943; F = 24.229; n = 8)			
Nicotine	.013 (± 1.089)	.012	.991
CGA	4.595 (± 6.328)	.726	.543
Rutin	1.048 (± .732)	1.432	.288
DTG	.023 (± .056)	.413	.719
TPI	-17.758 (± 76.294)	-.233	.837

Table 2: The influence of silencing herbivory-regulated proteins on larval performance. The associations among larval biomass, photosynthetic capacity, levels of RuBPCase and defense metabolites were determined by multiple regression analysis. The analysis was performed in two groups. In group 1, mean larval biomass was regressed against mean values of photosynthetic rate (600µmol CO_2 mol^{-1}) and RuBPCase contents; in group 2, mean larval biomass was regressed against mean OS-elicited concentrations of nicotine, CGA, rutin, DTG, TPI and larval midgut protease activity for all VIGS lines. All mean values were of five biological replicates.

Independent variables	Co-efficient (± SE)	t-Value	P-Value
Group 1 (R^2 = .345; adjusted R^2 = .083; F = 1.315; n= 8)			
RuBPCase level	38.78 (± 23.912)	1.622	.165
Photosynthesis	-8.051(± 7.386)	-1.09	.325
Group 2 (R^2 = 1.0; adjusted R^2 = .998; F = 724.105; n= 8)			
Nicotine	-64.994 (± 9.809)	-6.626	.095
CGA	-144.086 (± 56.55)	-2.548	.238
Rutin	2033.424 (± 98.785)	20.584	.03
DTG	-31.435 (± 1.095)	-28.713	**.0222**
TPI	31.537 (± .634)	49.751	**.0128**
Gut protease activity	-31.853 (± 2.797)	-11.387	**.0558**

Proteomic analysis of M. sexta midgut proteins

Midgut proteins from larvae reared on EV, *Nacad-, Nacpn*-, and *Nagmps*-silenced plants were separated by 2-DE and analyzed. We selected these lines because the larvae reared on these lines represented the range of effects observed in larval mass: similar (*Nacad*), greater (*Nacpn*), and less (*Nagmps*) compared to larvae reared on EV plants. In addition, these lines also encompassed the range of effects on photosynthetic rates and growth observed in the analysis.

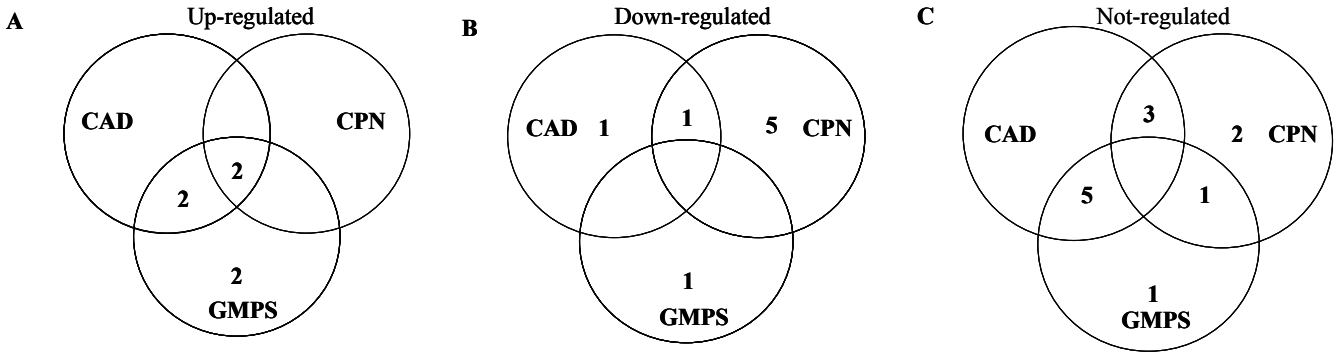

Fig. 3: Midgut (gut tissue + lumen content) proteins from *M. sexta* larvae that fed on EV, *Nacad-, Nagmps*-, and *Nacpn*-silenced plants were analyzed by 2-D PAGE (see **Fig. S3**) and the number of **A)** up, **B)** down, or **C)** not regulated protein spots compared to those in the midgut extracts of larvae that fed on EV plants are given. The entire experiment was repeated with the same results. See **Table S4** for the putative identities of the proteins.

The replicated analysis of midgut extracts from larvae reared on *Nacad*-silenced plants revealed 6 differentially regulated spots; of these, 2 occurred at the same levels as in the samples from *Nagmps*- and *Nacpn*-reared larvae. Comparing 2-DE images (**Fig. S5**) of *Nagmps* with EV midgut samples, we found 6 spots in *Nagmps*-silenced plants with increased protein levels and 1 spot with reduced protein levels (**Fig. 4A, B**); 4 of the 6 up-regulated spots in the *Nagmps* profile were unchanged in the *Nacad* profile (**Fig. 4C**). *Nacpn* midgut samples revealed down-regulation of 6 proteins (**Fig. 4B**). Of a total of 14 accumulated proteins, 8 spots of *Nacad*, 7 spots of *Nagmps*, and 6 spots of *Nacpn* had the same protein levels as EV plants (**Fig. 4C**). Differentially accumulated proteins were analyzed with the

Swissprot and NCBI insect- and plant-database. Although the identified proteins were mainly from insects, a few plant and bacterial proteins were also present **(Table S4)**. Up-regulation of stress-related proteins (TCP1 complex, guanylylcyclase88E, etc.) suggests that different diets affected the defense response of *M. sexta*.

Discussion

The *N. attenuata* genes selected for silencing and functional analysis in this study were categorized according to their influence on the two main dependent variables of this insect-plant interaction: photosynthetic performance (reduced or unchanged compared to EV plants) and *M. sexta* performance (reduced, unchanged, and more larval mass compared to larvae that fed on EV plants). To understand how these seven regulated proteins influence insect performance, we analyzed the most abundant secondary metabolites of *N. attenuata,* namely nicotine, CGA, rutin, DTG, and TPI, and the level of RuBPCase, the most abundant dietary protein. The data from control and induced plants were expressed in the currency relevant to the consumer (as a percentage of leaf mass) and the producer (as a whole-plant pool) (27). Silencing proteins that are up-regulated after herbivore attack (*Naatpe*, *Nacad*, *Nacpn*, and *Natk*) benefited or had no effect on *M. sexta* performance. Silencing genes that code for proteins that are down-regulated in response to herbivore attack (*Nagpdh*, *Nagmps*, and *Naoep*), on the other hand, had either adverse effects or no effect on larval performance. The effects on larval growth were significantly larger than those observed when known defense compounds in *N. attenuata* such as TPIs and nicotine are silenced (13, 14). Silencing the accumulation of the differentially regulated proteins in *N. attenuata* created diet conditions which affected the midgut protein profiles of *M. sexta*. The results demonstrate the utility of an unbiased "ask-the-proteome" approach to understand gene and protein function at an organismic level in a plant-herbivore interaction. In the following, we consider the effects of gene silencing on the plant-insect interaction, first from the plant's perspective and then from the insect's.

Regulated proteins influence plant growth, photosynthesis, and secondary metabolite production

When attacked by herbivores, a plant appears to choose between growth or defense responses (28). As expected, silencing genes that decreased photosynthetic capacity also

decreased growth. The photosynthetic rates of *Nacpn*-, *Nagmps*-, and *Natk*-silenced plants were significantly lower than those of EV plants at different CO_2 concentrations -- no surprise given their biochemical functions. However, for *atpe*-silenced plants, the connection to photosynthetic processes was less direct. Vacuolar *atpe* is known to be involved in basic cellular processes, such as ion and metabolite transport, and cell enlargement (21), but its role in photosynthesis is not understood. Of these 4 proteins that when silenced reduced photosynthesis and growth, two (*Nagmps* and *Naatpe*) were associated with decreases in RuPBCase pools (**Fig 1C**), and only one (*Nagmps*) is down-regulated during herbivore attack; the fact that the other three are naturally up-regulated suggests that they may help to minimize the well-established negative effects of herbivore damage on photosynthetic capacity (29).

Silencing genes that influence photosynthesis and growth are expected to also influence the plant's ability to produce and elicit secondary metabolite production and thereby indirectly influence herbivore resistance. The accumulation of secondary metabolites is thought to reflect the C-N balance of the plant (28), and whole-plant pools of C-intensive metabolites, rutin and DTGs, but not CGA, closely tracked photosynthetic capacity and growth (**Fig. S4**). For example, silencing TK dramatically impaired photosynthetic capacity and decreased phenylpropanoid metabolism, as has been shown in cultivated tobacco (23). However, not all responses were consistent with the predictions of the C-N balance hypothesis. For example, *Naoep*-silenced plants, which had photosynthetic rates that did not differ from EV plants, had higher concentrations of both the C-rich compound, CGA, as well as the N-rich compound, nicotine.

Secondary metabolism is known to scale allometrically with plant size and be highly tissue specific (30) and the concentrations of an abundant secondary metabolite depends not only on the synthesis of that particular compound but also on the synthesis of other plant constituents (31). Therefore, we expressed the metabolite production of silenced plants in a currency of whole-plant pools as well as in a concentration currency. The effects of gene silencing on metabolite production expressed in a concentration currency were similar to the effects expressed in a whole-plant currency, except in pTV*atpe*-silenced plants; pTV*atpe*-silenced plants showed exactly the opposite trends of nicotine and CGA accumulation when expressed as a whole-plant pool. These results may reflect the greater effect of *Naatpe* silencing on the growth of shoots compared to roots, as nicotine is produced in roots and accumulates in shoots (29)**.**

Nitrogen exported from the roots of *N. sylvestris* in the form of glutamine is sufficient to account for the increase in nitrogen exported from roots to shoots as nicotine (32). A similar switch between glutamine and nicotine exports may have resulted in increased nicotine levels in *Nagmps*-silenced plants. By manipulating *Nagmps*, we uncovered a difference in how plants allocate N to N-intensive defense metabolites. Despite the likely N-limited growth of *Nagmps*-silenced plants, the constitutive level of nicotine was similar (whole-plant basis) or higher (concentration basis) compared to EV plants, but in both cases significantly higher after elicitation**.** In contrast, constitutive and induced levels of another N-demanding inducible defense compound, TPI, were significantly lower (constitutive and induced) measured in both whole-plant as well as concentration currencies. Though *Nagmps*-silenced plants have low constitutive TPI levels, the increases in TPI activity after elicitation in *Nagmps*-silenced plants were stronger than they were in EV plants. Taken together, these results suggest that *gmps* helps orchestrate the allocation of N to N-intensive defenses. However, figuring out how it does so will require additional work.

Regulated proteins influence the performance of M. sexta

The vast majority of research into plant traits that influence host quality for herbivores has focused on secondary metabolites and has evaluated the impact of individual compounds on herbivore performance. However, recent research results have demonstrated that synergistic and antagonistic interactions among primary and secondary metabolites are crucial in determining host quality (33). These interactions may be particularly important for specialist herbivores such as *M. sexta* that have adapted to the defenses of their native host. In such cases, variation in the primary metabolites which provide the nutritional resources required to detoxify a plant's defenses may be more important than differences in defense concentrations (34). The results from this work are consistent with this view.

In previous work, we found that silencing the expression of *N. attenuata*'s *putrescine N-methyl transferase* (*pmt*), a key enzyme in nicotine biosynthesis, inhibited nicotine production and increased the performance of *M. sexta* larvae (13); however, in the present study, the substantial variation in nicotine accumulation after silencing *Naoep*, *Naatpe*, and *Nagpdh* **(Fig. 2E)** did not significantly influence larval performance, suggesting that other compounds affected caterpillar performance. Similar results were found for TPI accumulation, another established defense in *N. attenuata* (14), after the expression of *Naatpe* and *Nagmps* was silenced. Moreover, across all lines of this study, TPI activity was positively rather than negatively correlated with larval performance, as would be expected for an

established defense. To counteract the ingestion of TPIs, insects are known to produce TPI-resistant proteases (35, 36); moreover, high levels of dietary protein are known to negate the effects of TPI ingestion on larval growth (37) and to augment the ability of insects to detoxify secondary metabolites (34). However, in our study relationships among the activity of plant TPIs and gut serine protease activity and among RuBPCase levels and caterpillar growth were not consistent across different lines. In summary, the variation in herbivore performance on these silenced plants was not related to the variation in known defenses, and from this we infer that other, yet-to-be-described metabolites were more important for herbivore performance.

Because phenolics are generally thought to function as defenses, it was surprising to find a strong positive correlation between rutin concentrations and larval performance. Deboer and Hanson (38) had previously reported that rutin stimulated feeding in *M. sexta,* but Stamp and Horwath (1992) (39) found that *M. sexta* larvae gained less weight on artificial diets supplemented with rutin. Understanding the importance of rutin will require genetically manipulating plants that precisely target rutin accumulation.

The strongest evidence for the defensive function of a secondary metabolite was found in the variation in DTG levels that resulted from gene silencing **(Fig. 2F).** DTGs were implicated as potent resistance traits against *M. sexta* larvae in a multi-species comparison (40) and, more recently, in *N. attenuata* plants by silencing the source of the geranylgeranyl diphosphates (GGPPs) required for geranyllinalool biosynthesis, a key intermediate in DTG biosynthesis (41). Reductions in DTG concentrations in *Nacpn*- and *Natk*-silenced plants and a strong negative correlation between DTG and caterpillar biomass across all lines suggests that DTGs are more important than nicotine and TPI in influencing larval performance.

We used an unbiased analysis of insect midgut proteome to determine if *M. sexta* larvae adjusted their digestive physiologies to cope with variation in host nutritional quality. The gut protein profiles of larvae that fed on *Nacpn*-, *Nagmps*-, and *Nacad*-silenced plants were compared with the gut protein profiles of larvae that fed on EV plants. A comparative analysis of the 2-DE profile of larval midgut proteins suggested that silencing different candidate genes in host plants affects *M. sexta*'s mid-gut proteome in distinct ways **(Fig. 4).** The low body mass of pTV*gmps*-fed larvae indicated suboptimal nutritive conditions, which correlated with the largest number of up-regulated proteins. The high body mass of pTV*cpn*-fed larvae indicated optimal nutritive conditions compared to pTV*gmps*-fed larvae, which correlated with the smallest number of up-regulated proteins. Opposite trends were found in

down-regulated protein spots: the largest numbers of proteins were down-regulated in caterpillars that fed on pTV*cpn* plants and the smallest in caterpillars that fed on pTV*gmps* plants. Therefore, the up-regulation of a number of defense-related proteins may be necessary for insects to adapt to suboptimal diets.

Though there is no difference in caterpillar body mass between pTV*cad*-fed caterpillars and EV-fed caterpillars, the up-regulation of eight protein spots indicated that these proteins were necessary to maintain caterpillar performance. We found several protein spots in GMPS, CPN and CAD samples which did not change their accumulation patterns when compared to EV samples. In comparisons across lines, however -- namely among GMPS-CPN, GMPS-CAD, and CPN-CAD -- changed accumulation patterns were apparent. This observation emphasizes the diet-specific accumulation and the dynamism of midgut proteins in *M. sexta*. Clearly, the ability to manipulate the expression of these proteins in larvae will be required before their functional significance can be fully understood. The recent report of the silencing of *Helicoverpa armigera* larval genes by expressing RNAi constructs containing larval genes in the larvae's host plant represents a means of using the established transformation systems in plants to manipulate the expression of the insects' counter-defense responses (42).

Our "ask-the-proteome" approach posits that after herbivory, plant proteins are selectively up- and down-regulated in an adaptive manner. Apart from their known biochemical functions, candidate proteins play direct and/or indirect roles in plant-herbivore interaction by influencing the metabolic framework in both plants and insects. We found that silencing some of the candidate proteins has stronger effects on caterpillar performance than silencing the expression of known defense proteins. From the multiple regression analyses, we infer that herbivore performance was most affected by DTG concentrations; however, that variation in DTG could not account for all the variation in larval performance suggests that other unknown defenses and/or synergistic interactions among metabolites remain to be discovered. In addition, the analysis identified new controls over the regulation of growth and defense in *N. attenuata*. For example, the control of N-allocation over inducible and constitutive nicotine and TPI production in *Nagmps*-silenced plants and the significant increase in growth of *Nacad*-silenced plants despite unchanged photosynthetic rate illustrate a new mode of metabolite allocation. Larval performance on *Nacpn*-silenced plants and the lack of a correlation between TPI and gut protease accumulation raise important questions about how the chaperonic activity of *Nacpn* contributes to DTG production and influences other

defense traits. In addition, the gut protein profiles of larvae suggest that herbivores are as metabolically dynamic as their host plants during the interaction.

If we are sufficiently forward-thinking to ignore the gene annotations, to silence these regulated responses in ways that don't dramatically influence growth, and then to ask the community of herbivores that naturally attack plants if the plant is more resistant to or tolerant of herbivore attack, we will undoubtedly learn much that is new about how plants survive in the real world. An unbiased "ask-the-herbivore" approach can also be a potent method to infer, on the one hand, the strategies used by plants against herbivores and, on the other hand, the strategies insects use to counter plant defenses. While the insights gained will require additional focused research to fully understand, unbiased holistic analyses offer an interesting tool with which to explore dynamic biotic interactions.

Materials and Methods

Virus induced gene silencing (VIGS)

Previously reported sequences of *N. attenuata*'s *atpe* (DQ682456), *cad* (EF679773), *cpn* (DQ682457), *gpdh* (DQ682459), *gmps* (DQ682458), *oep* (DQ682462), and *tk* (DQ682468) were used to make the Tobacco Rattle Virus (TRV)-based construct for virus-induced gene silencing (VIGS) as described previously (20). Three-week-old *N. attenuata* plants were inoculated with *Agrobacterium tumefaciens* and transformed with pTV-candidate genes or pTV-*phytoene desaturase* (*pds*) or empty vector (EV) constructs (20). Growth conditions and experimental set-up were as described in (18).

Transcript quantification

The total RNA was extracted from first stem leaves of transformed plants and 2 μg of total RNA from each sample was used to synthesize cDNA using a Superscript first strand synthesis system and the transcript levels of candidate genes in VIGS-silenced plants was analyzed by semi-quantitative RT-PCR as described in (43).

CO_2-exchange and growth

Net photosynthetic rates and intercellular CO_2 concentrations were measured on five-week-old EV and VIGS-silenced, glasshouse-grown plants under saturating light (~1200μM PAR m^{-2} s^{-1}) using Li-Cor 6400 Portable Photosynthesis System (LiCor Biosciences, Lincoln, NE, USA). Net photosynthesis rates were measured in at least five replicate plants, each at

three different CO_2 concentrations, namely, 200, 400, and 600 μmol CO_2 mol^{-1}. Plants were harvested at seven weeks and dried in the oven at 60°C for 48 h before weighing.

Secondary metabolites

To examine the effect of gene silencing on secondary metabolite production, leaves of EV and VIGS-silenced plants were sampled before and 4 days after elicitation with *M. sexta* larval oral secretions (OS): puncture wounds were created with a fabric pattern wheel and larval OS were immediately applied to the puncture wounds (20μl). OS were collected from fourth- and fifth-instar larvae that fed on *N. attenuata* WT plants. 100 mg of leaf lamina was used to analyze the levels of nicotine, DTGs, CGA, and rutin by high-performance liquid chromatography as previously described (44).

M. sexta performance

A single neonate of *M. sexta* larvae was placed on the first stem leaf of EV and VIGS-silenced plants. 30 larvae were weighed at 4, 8, and 12 days, at which time they had reached third to fourth instars. The midguts of these larvae were analyzed for protein profiles and protease activity.

M. sexta midgut protease activity

Five 4th-instar larval midguts (gut tissue + lumen content) were pooled and analyzed for gut protease activity as described (45). To obtain protease profiles of gut extracts, an equal concentration of protein extract (10μg) isolated from 100mg tissue was resolved on a 10% native polyacrylamide gel electrophoresis (PAGE). Proteases were visualized using the gel X-ray film contact print technique (46).

Total leaf proteins and TPI assays

Leaves from VIGS plants were harvested 4 days after OS elicitation. Soluble protein was extracted and quantified, and levels of constitutive and induced TPI activity were determined by radial diffusion assay with bovine trypsin as described in (47).

Protein extraction, separation by 1-DE and 2-DE, and analysis

Total protein was extracted from leaf and midgut tissue (pooled gut tissue + lumen content of five 4th-instar larvae) with a modified phenolic extraction procedure and quantified with 2-DE Quant kit (43). 12% SDS-PAGE was carried out for 1DE analysis of leaf proteins.

Gut proteins were subjected to 2-DE analysis. For first-dimension separation, 11 cm IPG strips pH 3-10 NL (Biorad, Munich, Germany) were rehydrated with 200 µg of protein in the final volume of 200 µl with rehydration buffer, for 16 h at room temperature. The proteins were focused on a Protean IEF Cell (Biorad, Munich, Germany) at 20°C, rapid voltage ramping, 32000 volt h, and 50 µA current per IPG strip. (43). Gel images were created using a GS-800 calibrated densitometer (Biorad, Munich, Germany). Spot detection, indexing, matching, normalization, and quantification were done using the PDQuest v7.3.1 software (Biorad, Munich, Germany). Protein spots of interest were excised and digested and analyzed by MALDI-TOF and QTOF as described in (43).

Statistical analysis

Data were analyzed by ANOVA and multiple regression models with StatView (Abacus Concepts, Berkeley, CA, USA).

Acknowledgments

APG acknowledges the Alexander von Humboldt Foundation for the fellowship. We thank K. Gase and A. Wissgott for making VIGS constructs, A. Muck and A. Svatoš for mass spectrometric analysis, T. Krügel, A. Weber, and A. Schünzel for growing the plants, E. Wheeler for editorial assistance and O. Mittapalli for comments.

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3.3 Manuscript III

Effect of decreased photosynthetic rates on herbivore performance in *Nicotiana attenuata*

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§This work is supported by the Max Planck Society

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Running title: Silencing *RCA* and *RuBPCase* in *N. attenuata*

ABSTRACT

Insect herbivory down-regulates photosynthetic proteins in *Nicotiana attenuata*. The consequences of decreased photosynthesis and its effect on herbivores were analyzed in *N. attenuata* plants, which had been independently transformed with RuBPCase activase (*RCA*) and RuBPCase (*RUB*). Silencing *RCA* and *RUB* reduced photosynthesis by 40-50% in rosette-stage plants, and at the whole-plant level, by 50-55% in *RCA*-silenced plants and 25% in *RUB*-silenced plants. The growth of *RCA*- and *RUB*-silenced plants is consistent with the rate of photosynthesis. *Spodoptera littoralis* larvae grew faster when fed on *RCA*-silenced plants, which is consistent with the decreased accumulation of trypsin protease inhibitors (TPIs) and diterpene glycosides (DTGs). In contrast, larval growth on *RUB*-silenced plants was similar to that of those that fed on empty vector-(EV) transformed plants, which is consistent with the unaltered production of TPIs and DTGs. *RCA*-silenced plants were impaired in their accumulation of jasmonate (JA)-Ile/Leu but RUB-silenced plants were not. *RCA*-silenced plants complemented with external Ile were unable to increase their JA-Ile/Leu levels. The transcript accumulation of JAR4/6, the key enzyme for JA-Ile conjugation, in irRCA plants, is similar to that in EV-transformed plants. We conclude that *N. attenuata*'s photosynthetic capacity is larger than what is required to maintain defense responses. The results from the complementation experiments, transcription profiles of *JAR*4/6, and total photosynthesis rate suggest that JA-Ile levels in *RCA*-silenced plants are influenced by carbon availability than the size of Ile pool and lower activity of conjugating JA with Ile. Total photosynthetic capacity influences the production of defense metabolites and the resistance of *N. attenuata* plants.

Key words: Dietary protein, fitness, resource allocation*, Spodoptera littoralis.*

Introduction

The molecular interaction between the specialist herbivore *Manduca sexta* and its natural host *Nicotiana attenuata* has been well studied at transcriptomic and proteomic levels (Schmidt et al., 2005; Giri et al., 2006). In a comparative proteomic-transcriptomic study of *N. attenuata* plants, over 90 meaningful amino acid sequences were found to be differentially regulated. Proteins that increased were involved in primary metabolism, defense, transcriptional and translational regulation; those that decreased were involved in photosynthesis (Giri et al., 2006). An induced plant is known to reduce its photosynthetic capacity while increasing the production and accumulation of defense-related compounds (Walling, 2000; Hermsmeier et al., 2001; Kessler and Baldwin, 2002; Hahlbrock et al., 2003). Under such conditions plants must grow rapidly to compete and simultaneously maintain the defenses necessary to survive in environments with herbivores (Herms and Mattson, 1992). The dramatic up-regulation of defense metabolites likely requires metabolic adjustments in plant growth and reproduction (Halitschke et al., 2003; Reymond et al., 2004; Ralph et al., 2006). However, reductions in plant growth can also be understood as part of a plant's defense strategy, as they limit the availability of food and nutrition to the feeding insect (Hermsmeier et al., 2001; Hahlbrock et al., 2003). Allocating resources for resistance mechanisms is believed to reduce the availability of resources for growth; hence resistance is costly in terms of plant growth and fitness (Heil and Baldwin, 2002). However, very little is known about how plants optimize their carbon allocation when attacked by herbivores. A recent study proposes that the 'acclimatory response' of a plant is responsible for tuning the balance between carbon demand and supply to optimize plant's growing capacity (Smith and Stitt, 2007). For example, by allocating newly assimilated carbon to roots rather than transporting it to young leaves, plants exhibit a novel defense strategy for tolerating herbivore attack (Schwachtje et al., 2006). Manipulating photosynthesis is likely the best way to investigate the carbon allocation strategy of plants when they are attacked by herbivores.

In our *N. attenuata*-*M. sexta* interaction study, we observed a substantial change in the accumulation of RuBPCase activase (RCA) and RuBPCase at transcript and protein levels after wounding (W) and applying *M. sexta* regurgitate (OS) to wounds, a procedure which elicits *M. sexta*-specific responses (Halitschke et al., 2001; Roda et al., 2004; Giri et al., 2006). The key role of *RCA* is to modulate the activity of RUBPCase, the major photosynthetic protein, by removing the inhibitory sugar phosphates from the active site of the enzyme, whether or not the enzyme is carbamylated (Portis, 2003). The effect of *RCA* on

photosynthesis and growth has been well studied in *Arabidopsis thaliana* and *Nicotiana tabacum*. In *A. thaliana,* moderate reductions of *RCA* affect plant growth by decreasing above-ground biomass and leaf area (Ilyin et al., 2005). However, *N. tabacum* plants transformed with *RCA* expressed in an antisense orientation are slow growing as compared to wild type (WT) plants eventually attained the same height and number of leaves (He et al., 1997). In addition, as a member of the AAA^+ (ATPases associated with a variety of cellular Activities) protein family, RCA can also possess diverse cellular-processing abilities (Ogura and Wilkinson, 2001). For example, during sudden exposure to heat stress, *RCA* assumes the role of a chaperon in association with thylakoid-bound ribosomes, possibly protecting the thylakoid-associated protein synthesis machinery (Rokka et al., 2001). Therefore, in *N. attenuata, RCA* is one of the major candidates thought to orchestrate the down-regulation of photosynthesis-related genes in response to stress, which, in turn, allows a plant to optimize its allocation of resources to growth, reproduction, and defense. RuBPCase, a major photosynthetic protein, accounts for 40% of total leaf protein (Taiz and Zeiger, 1998) and is a main dietary protein for the herbivores (Felton, 2005). The effect of RuBPCase silencing on photosynthesis, growth, and metabolite production has been well studied in *N. tabacum*. The decreased expression of RuBPCase strongly reduces the photosynthesis rate in tobacco. However, the growth of RuBPCase silenced plants was reduced in parallel with photosynthesis only under high nitrogen (N); when N was limited, growth was hardly affected (Fichtner et al., 1993). A decrease of RuBPCase activity in *N. tabacum* inhibited nitrate reductase activity and nitrate accumulation; in addition, RuBPCase silencing also decreases the levels of amino acid, chlorogenic acid, and nicotine, which are the major carbon- and nitrogen-rich secondary metabolites in tobacco leaves. Moreover, the regulation of RCA and RuBPCase in response to UV-B exposure, ozone, heat stress, and drought (Pelloux et al., 2001; Liu et al., 2002; Bota et al., 2004; Demirevska-Kepova et al., 2005) in different plant systems, suggested their involvement in stress-related functions.

Though it is believed that when attacked by herbivores, a plant chooses between growth and defense responses (Herms and Mattson, 1992), Smith and Stitt (2007) suggested that the interplay between resistance and growth is not in a "straight competition" when growth is reduced due to direct carbon limitation. Therefore, manipulation of photosynthesis by silencing the expression of *RCA* and *RUB* in *N. attenuata* can reveal how herbivore resistance is influenced by reduced carbon resources. In our preliminary study in *N. attenuata*, silencing the expression of *Narca* by virus-induced gene silencing (VIGS) caused the plant's photosynthetic ability and biomass to decrease (Giri et al., 2006). To understand that how *N.*

attenuata plants reorganize resources after a herbivory-induced decrease in photosynthesis and how they acclimatize, we manipulated the photosynthesis of *N. attenuata* by independently silencing the expression of their *RCA* and *RUB* genes. Transformed plants were evaluated for their levels of secondary metabolites, dietary proteins, jasmonate signaling, and their resistance to generalist (*Spodoptera littoralis*) herbivore compared to empty vector- (EV) transformed plants.

RESULTS

Stable transformation of *RCA* and *RUB* in *N. attenuata*

To generate stable transformants, we silenced *Narca* and *Narub* by *Agrobacterium*-mediated transformation (Krugel et al., 2002) using a pRESC transformation vector containing an inverted-repeat fragment of 268bp *Narca* gene and a 316 bp fragment of *Narub* gene in antisense orientation. Using Southern hybridization, we identified two independently transformed *RCA* lines (irRCA lines 1 and 2) and one *RUB* line (asRUB) with a single transgene insertion (Fig. S1). These lines were used for all further experiments. *N. attenuata* plants transformed with an empty vector (EV) served as control.

Expression of *Narca* and *Narub* in Transformed Plants

The constitutive expression of *RCA* and *RUB* in transformed plants was analyzed by quantitative RT-PCR. The accumulation of *RCA* transcripts in untreated leaves of *N. attenuata* plants was significantly lower (by 40%) in irRCA lines (1 and 2) and the accumulation of *RUB* transcripts of untreated leaves of asRUB plants was 50% that of untreated EV plants (**Fig.1A and B**). The accumulation of RCA transcripts in asRUB plants and the accumulation of RUB transcripts in irRCA plants are not significantly different than EV plants.

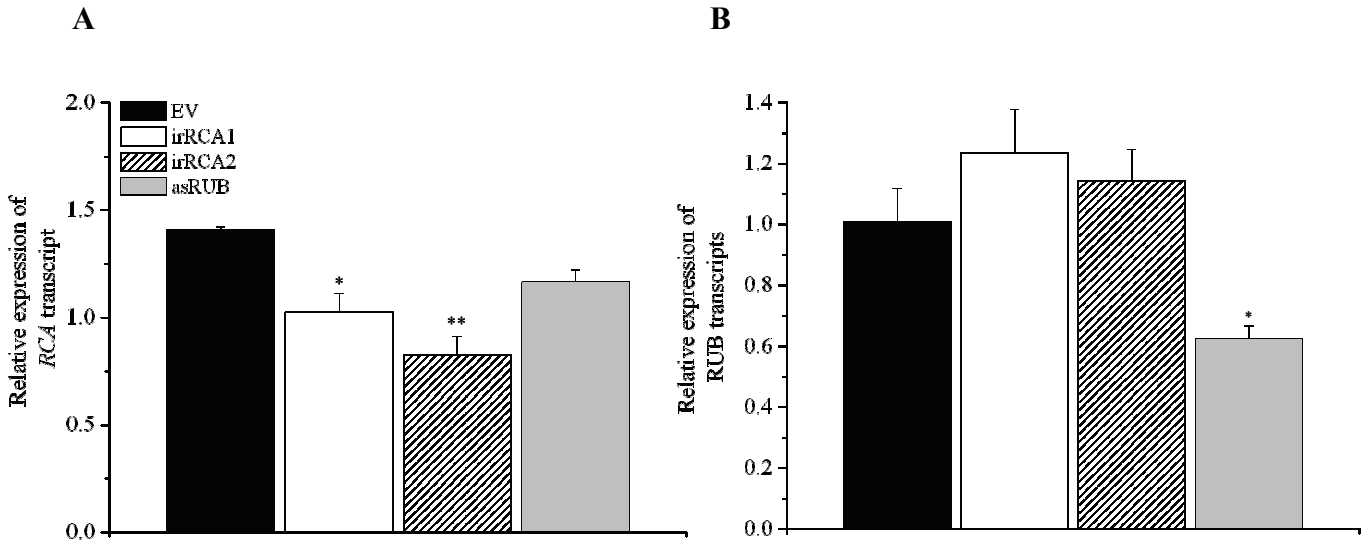


Figure 1. Transcript levels of *RCA* and *RUB* Are Suppressed after Silencing *Narca* and *Narub* in *N. attenuata*. Transcript levels of *Narca* and *Narub* were analyzed in untreated EV, irRCA, and asRUB plants by quantitative RT-PCR. The expression levels of *Narca* and *Narub* were normalized to the expression levels of an unregulated gene, *actin*. **(A)** Untreated irRCA plants accumulated significantly fewer *RCA* transcripts (40- 42%) compared to EV-transformed plants; RUB-silenced plants also accumulated fewer, but not significantly. **(B)** Untreated asRUB plants accumulated fewer *RUB* transcripts (~50%) compared to EV plants. irRCA plants also accumulated fewer RUB transcripts. Values are means of 3 biological replicates. Asterisks indicate a significant difference at P <0.05 (*).

Photosynthesis and Growth in Transformed Plants

The photosynthetic rates of independently transformed irRCA and asRUB plants were lower than those of EV-transformed plants. Under ambient CO_2 concentration (400µmol mol^{-1}) and a different light regime (0-2000 µmol m^{-2} s^{-1}), irRCA and asRUB plants had only 50% of the photosynthetic capacity of EV plants (Fig. 2A), while under saturating light and different CO_2 concentrations (0-650 µmol mol^{-1}) they had 80% (Fig. S2). EV-transformed plants showed efficient photosynthesis at ambient CO_2 as well as under limited CO_2 concentrations (200 µmol mol^{-1}); irRCA and asRUB plants had significantly lower photosynthetic rates even at higher (650µm mol^{-1}) CO_2 concentrations (ANOVA; $F_{3,16}$= 5.77; P_{irRCA1}= .022; P_{irRCA2}= .007; and $P_{asRUB} < .001$) compared to EV-transformed plants. The photosynthesis rates of irRCA and asRUB plants are not significantly different from each other (ANOVA; $F_{3,16}$= 5.77; P > .1). At the whole plant level, silencing RCA reduced the photosynthesis rate by 50-55% but silencing RUB only reduced it by 25% (**Fig. 2B**)

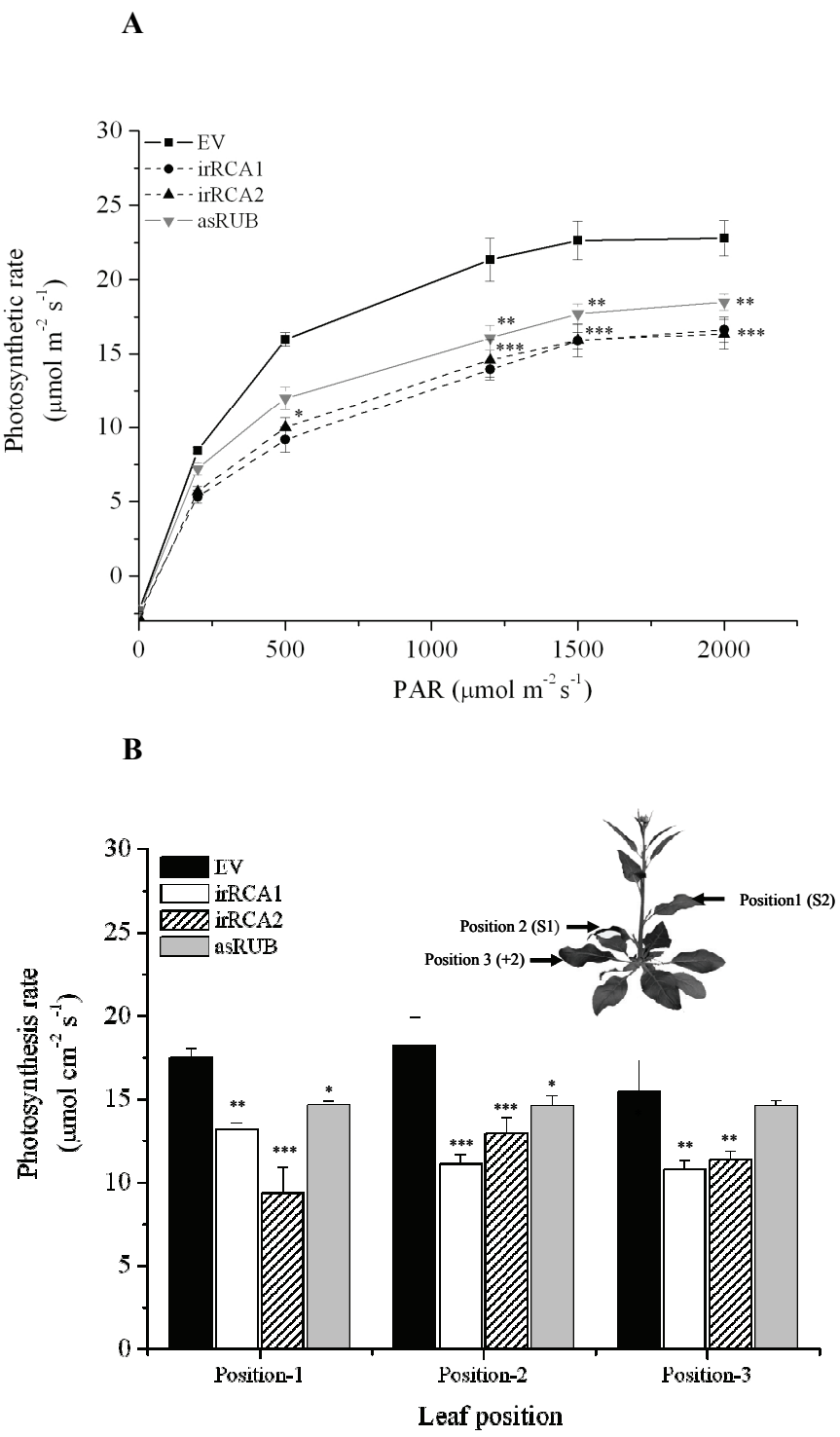


Figure 2. Silencing *RCA* and *RUB* Decreases the Photosynthetic Rate of *N. attenuata* at the Whole Plant Level (A) A light curve was generated at the CO_2 concentration of 400 µmol mol^{-1} and at six different points of irradiance (PAR), namely 0, 200, 500, 1200, 1500, and 2000 µmol m^{-2} s^{-1} at the rosette stage of EV, irRCA (lines 1 and 2), and asRUB plants. The assimilation rates of irRCA and asRUB plants decreased 40-50% at different PAR. **(B)** CO_2 exchange was measured at the light intensity of 1200 µmol m^{-2} s^{-1} and optimum (400 µmol mol^{-1}) CO_2 concentration at three different leaf positions, namely position 1 (S2 leaf), position 2 (S1 leaf), and position 3 (+2 leaf) in EV, *RCA*- and *RUB* silenced plants. The assimilation rates of *RCA*- and *RUB*-silenced plants decreased at every position compared to those of EV plants. However, the decrease in position 3 of *RUB*-silenced plants was not significant. Values are means (±SEM) of five or four replicate plants from each genotype and each position. Asterisks indicate a significant difference at P <0.005 (*), P <0.005 (**) and $P \leq 0.0001$ (***).

Growth performance was estimated by measures of stalk length. Stalk lengths of irRCA plants (Fig. 3A) were significantly reduced (by 12-20) (ANOVA; $F_{3,36}$ = 18.268; P_{irRCA1}= .0006; P_{irRCA2} <.0001) after silencing the expression of *RCA*. Stalk lengths of asRUB plants were also significantly shorter (by 60%) at the beginning of measurements, but they showed a tendency to eventually catch up to those of EV-transformed plants (ANOVA; $F_{3,36}$ =

18.396; P_{asRUB}= .0003) (**Fig. 3A**). When irRCA plants were grown in competition with EV plants, their stalk lengths were about 40% shorter (paired t test; n=10; t= 5.81; P_{irRCA1} =.0003; t= 10.04; P_{irRCA2} <.0001); even after 48 days of germination, the lengths of RUB-silenced stalks did not catch up to those of EV- transformed plants remained 8% shorter (paired t test; n =10; t = 4.29; P_{asRUB} =.002) (**Fig. 3B**).

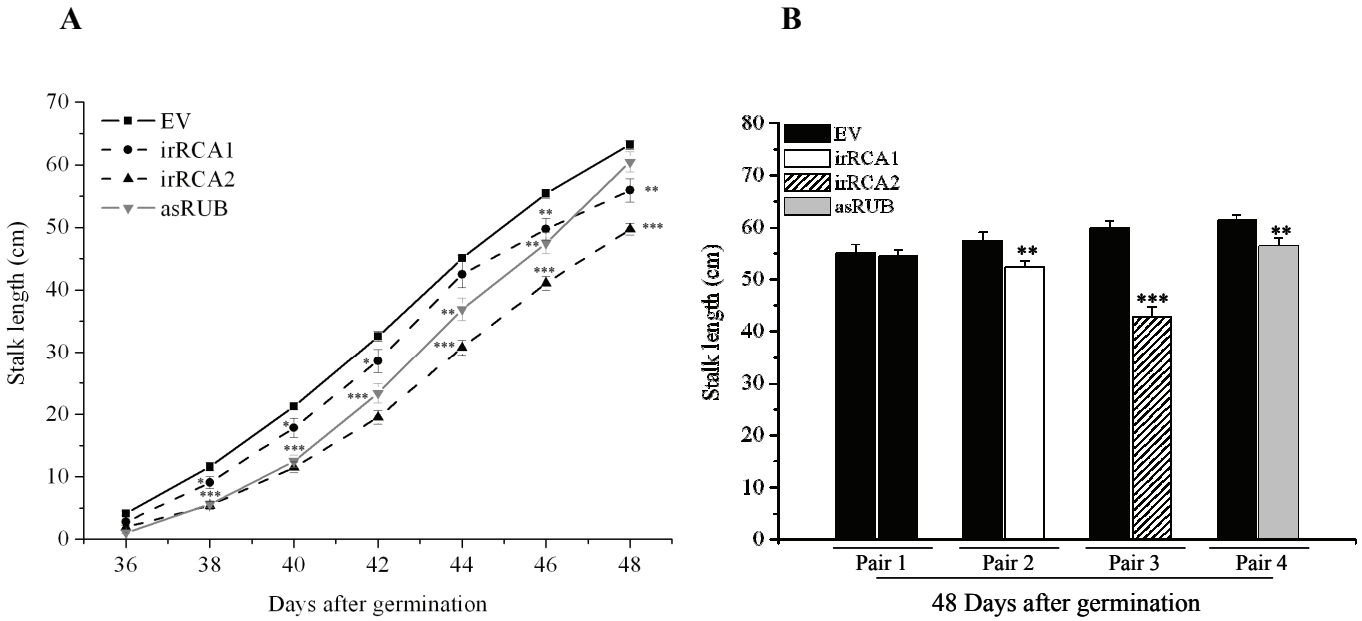


Figure 3. Decrease In CO_2 Assimilation Rate Impairs Growth in Both Single- and Competition-Grown *RCA*- and *RUB*-Silenced Plants. (A) The stalk lengths of singly-grown EV, irRCA, and asRUB plants were recorded for seven days. The stalk lengths of irRCA (lines 1 and 2) and asRUB plants were significantly less than those of EV plants**. (B)** The stalks lengths of irRCA and asRUB plants grown in competition with EV plants were significant decreased compared to the stalk lengths of EV plants which were also grown in competition with EV plants. Values are means (±SEM) of ten replicate plants from each genotype. Asterisks indicate a significant difference at P <0.005 (*), P <0.005 (**) and $P \leq 0.0001$ (***).

TPI Activity and DTG Accumulation in Transformed Plants

TPI activity decreased significantly (ANOVA; $F_{2,12} = 3.87$; $P_{irRCA1} = .04$; $P_{irRCA2} = .03$) after irRCA plants were induced with wounding (W) and the application of oral secretion (OS) . The accumulation of DTGs was significantly less at constitutive

(ANOVA; $F_{3,16}$ = 2.47; P_{irRCA1} = .04; P_{irRCA2}< .03) and induced (ANOVA; $F_{3,13}$ = 5.011; P_{irRCA1} = .05; P_{irRCA2}= .002) levels than in EV plants **(Fig. 4A and B),** but the accumulation of both TPIs and DTGs were similar in asRUB- and EV-transformed plants (TPI: ANOVA; $F_{1,8}$ = .56; P = .47; DTG: $F_{1,7}$ = 1.82; P = .22;) even after W+OS induction.

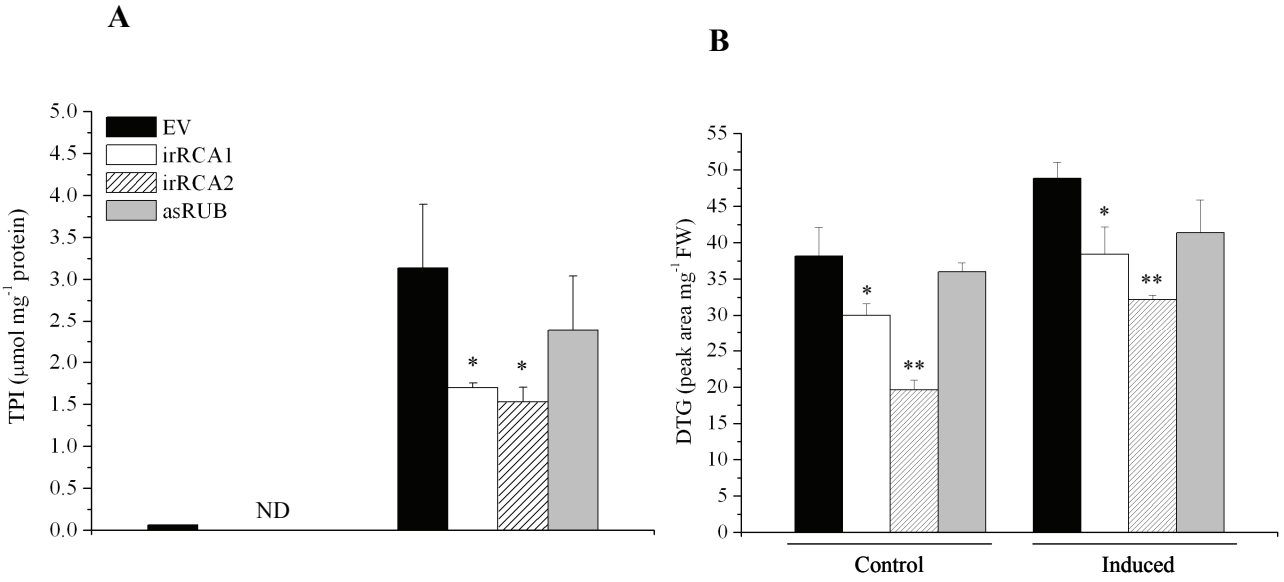


Figure 4. ***RCA*-Silenced Plants Accumulate Fewer DTGs and Trypsin Protease Inhibitors but *RUB*-Silenced and EV-Transformed Plants Accumulate Similar Amounts of DTGs and Trypsin Protease Inhibitors.** Rosette-stage leaves (+1) of irRCA, asRUB and EV plants were wounded with a pattern wheel and treated with 20µl of oral secretions (OS) of *M. sexta*. Tissues were harvested after 4 days of elicitation. Untreated leaves of each genotype served as control. *RCA*-silenced plants accumulate fewer **(A)** trypsin protease inhibitors and **(B)** DTGs than did EV-transformed plants. Values are means (±SEM) of five replicate plants from each genotype. Asterisks indicate a significant difference at P <0.005 (*), P <0.005 (**) and $P \leq 0.0001$ (***).

JA, JA-Ile, and *JAR*4/6 Accumulation in Transformed Plants

To find out why fewer defense metabolites accumulated in RCA-silenced plants, JA and JA-Ile were evaluated after W+OS treatment in all transformed plants **(Figs. 5A and 5B)**. JA accumulation in silenced plants is the same as in EV-transformed plants. However, the accumulation of JA-Ile irRCA plants significantly decreased 45 minutes after elicitation (ANOVA; $F_{3,16}$ = 5.143; P_{irRCA1}= .005; P_{irRCA2} = .05).

Decreased Ile pool can influence the accumulation of JA-Ile in irRCA plants. Therefore JA-Ile accumulation in RCA-silenced plants was also evaluated after these plants were treated with W+OS+Ile and compared with EV-transformed plants. RCA-silenced plants complemented with external Ile were unable to increase their JA-Ile/Leu levels, as were EV plants. (ANOVA; F2,9 = 4.154; P_{irRCA1}= .04; P_{irRCA2}= .02) **(Fig. 6A)**.

Transformed plants were elicited with W+OS and after 60 min of elicitation the expression of *JAR* 4/6 was analyzed. The expression of *JAR*4 was unaltered in transformed plants compared to EV plants and the expression of *JAR*6 was higher than that of EV plants **(Fig. 6B)**.

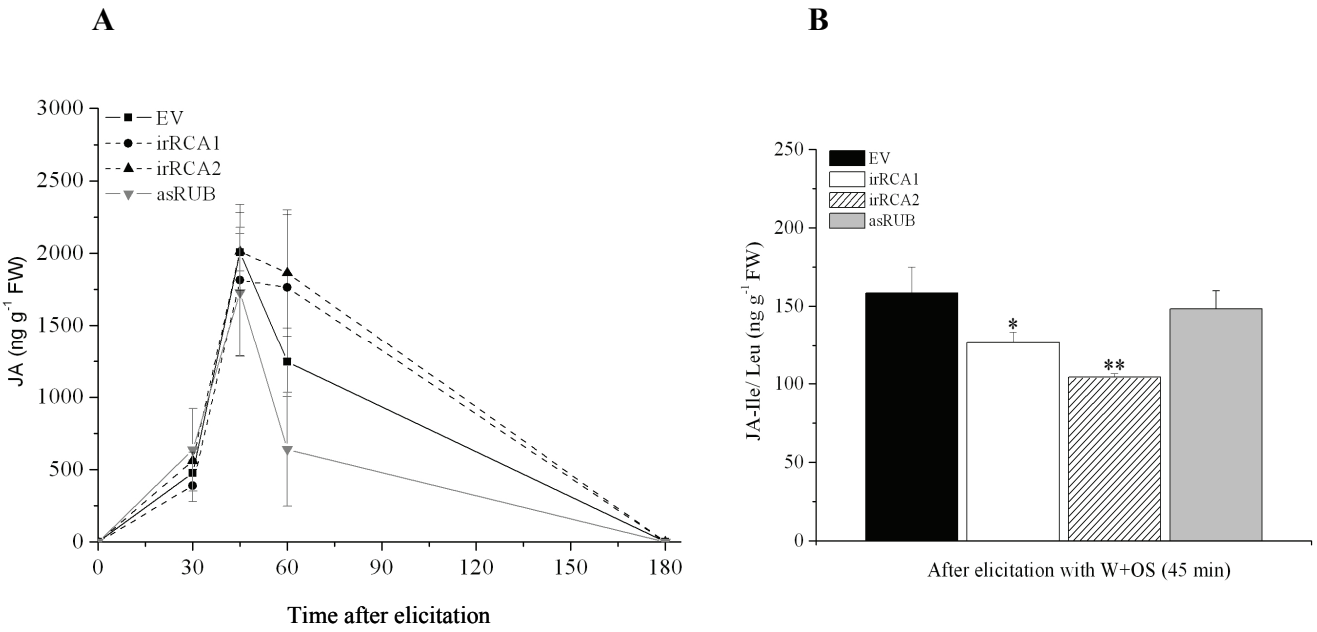
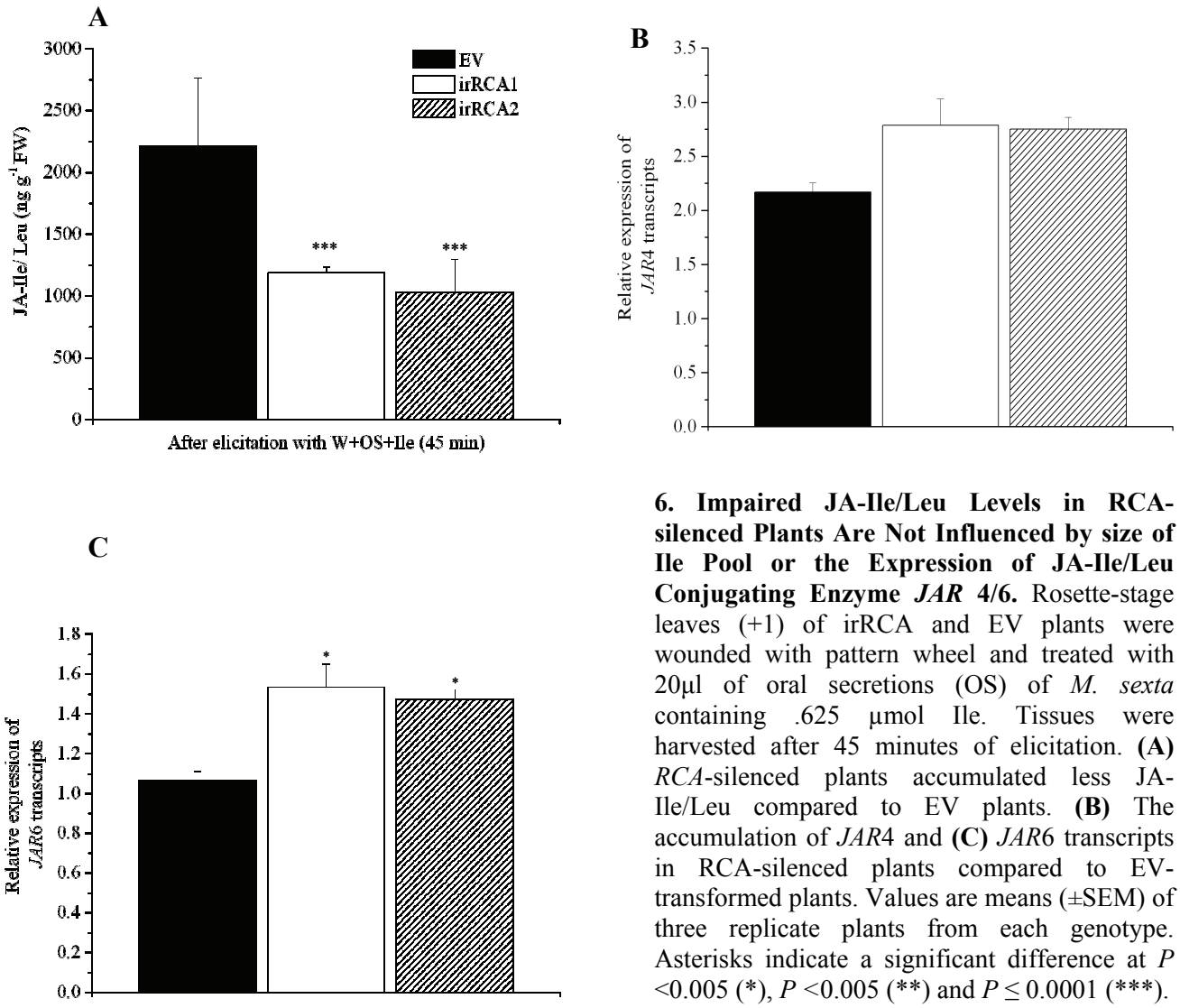


Figure 5. RCA-Silenced Plants Are Impaired in Their Accumulation of JA-Ile/Leu but Not JA; RUB-Silenced Plants and EV- Transformed Plants Did Not Differ in Their Accumulation of JA and JA-Ile/Leu . Rosette-stage leaves (+1) of irRCA, asRUB, and EV plants were wounded with a pattern wheel and treated with 20µl of oral secretions (OS) of *M. sexta*. Tissues were harvested after 0, 30, 45, 60, and 180 minutes of elicitation. *RCA*- and RUB-silenced plants accumulated similar amounts of **(A)** JA compared to EV-transformed plants. **(B)** RCA-silenced plants accumulated less JA-Ile/Leu but RUB-silenced plants and EV plants accumulate similar amounts 45 minutes after elicitation. Values are means (±SEM) of five replicate plants from each genotype. Asterisks indicate a significant difference at P <0.005 (*), P <0.005 (**) and $P \leq 0.0001$ (***).



6. Impaired JA-Ile/Leu Levels in RCA-silenced Plants Are Not Influenced by size of Ile Pool or the Expression of JA-Ile/Leu Conjugating Enzyme *JAR* 4/6. Rosette-stage leaves (+1) of irRCA and EV plants were wounded with pattern wheel and treated with 20µl of oral secretions (OS) of *M. sexta* containing .625 µmol Ile. Tissues were harvested after 45 minutes of elicitation. **(A)** *RCA*-silenced plants accumulated less JA-Ile/Leu compared to EV plants. **(B)** The accumulation of *JAR*4 and **(C)** *JAR*6 transcripts in RCA-silenced plants compared to EV-transformed plants. Values are means (±SEM) of three replicate plants from each genotype. Asterisks indicate a significant difference at $P < 0.005$ (*), $P < 0.005$ (**) and $P \leq 0.0001$ (***).

Performance of *S. littoralis* Larvae on Transformed Plants

To examine the effect of decreased photosynthesis on a generalist herbivore, the performance of *S. littoralis* larvae that fed on RCA-, RUB-, and EV-transformed plants was evaluated **(Fig. 7)**. *S. littoralis* larvae were grown on artificial diet for 6 days and then transferred to the rosette-stage leaves of EV, irRCA, and asRUB plants and allowed to feed for 12 days. *S. littoralis* larvae gained more body mass only when fed on irRCA plants compared with larvae that fed on EV plants (ANOVA; $F_{3,36} = 6.25$; $P_{irRCA1} = .04$; $P_{irRCA2} = .0014$; $P_{asRUB} = .86$).

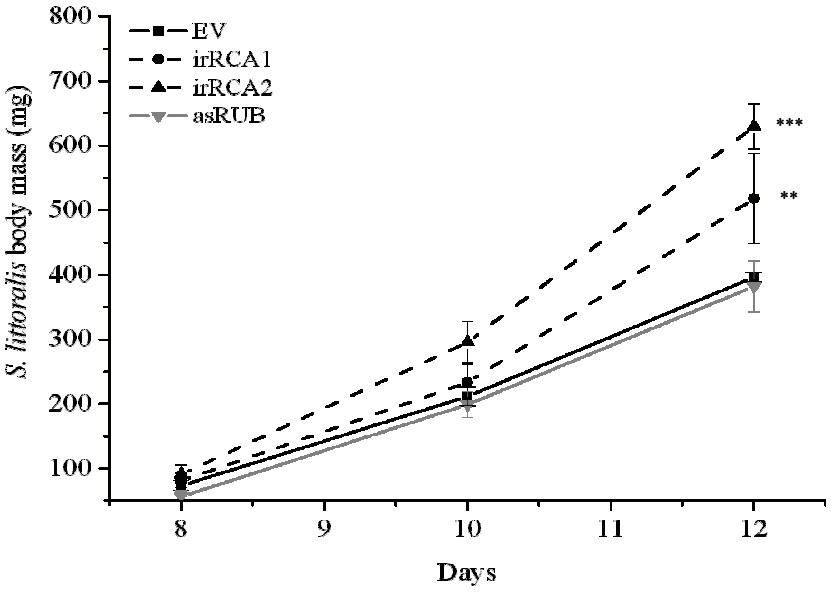


Figure 7. *S. littoralis* Performed Better Only on irRCA Plants, Not on asRUB Plants. Mass gain (mean± SEM) of **(A)** *S. littoralis* larvae on 12 replicate plants from EV, irRCA (lines 1 and 2), and asRUB genotypes were recorded. *S. littorallis* larvae grown on irRCA plants gained significantly more body mass than the larvae grown on *RUB*- and EV-transformed plants. Values are means (±SEM) of 12 caterpillars that fed on 12 replicate plants from each genotype. Asterisks indicate a significant difference at $P < 0.005$ (*), $P < 0.005$ (**) and $P \leq 0.0001$ (***).

RuBPCase Levels in Transformed Plants

RuBPCase, which contributes up to 40% of plants' total protein, is the major dietary protein source for insects (Johnson et al., 1996). He et al. have shown that silencing *RCA* increases RuBPCase content (He et al., 1997). In order to determine if silencing *RCA* and *RUB* influences the accumulation of large subunits of RuBPCase in *N. attenuata*, we performed a one-dimensional protein analysis of EV, irRCA, and asRUB plants **(Fig. 8)**. The amounts of RuBPCase increased 1-1.2-fold **(Fig. 8)** in irRCA plants but decreased 1.5-fold in asRUB plants compared to the amounts of RuBPCase in EV-transformed plants.

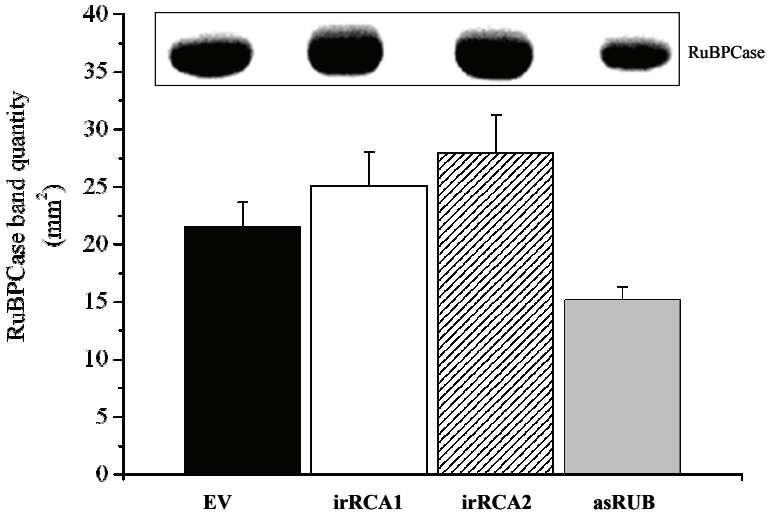


Figure 8. RUBPCase Accumulation Is Increased in irRCA Plants but Decreased in asRUB Plants. A) 1-D gel electrophoresis of large subunit of RuBPCase in irRCA plants compared to EV plants. **(B)** Area of RUBPCase large sub-unit bands as quantified in EV, irRCA, and asRUB (lines 1 and 2) plants by PD-Quest software revealed a ~1-1.2-fold increase in the amount of RUBPCase in irRCA plants, a 1.5-fold decrease in asRUB plants, compared to EV plants. Values are means (±SEM) of two replicate plants from each genotype.

DISCUSSION

Herbivore attack reduces a plant's photosynthetic capacity more than is expected given the canopy area removed by the herbivore (Zangerl et al., 2002), a result that points to herbivore-induced reductions in *RCA* and *RUB* as a potential explanation for the large decrease in the carbon assimilation rate that characterizes herbivore-attacked leaves. Silencing *RCA* and *RUB* expression reduces photosynthetic capacity in a number of different species (Fichtner et al., 1993; Hammond et al., 1998; Ilyin et al., 2005; Jin et al., 2006), but these transformed lines have not been characterized in the context of the complex changes that are elicited by herbivore attack (Hermsmeier et al., 2001; Schmidt et al., 2005). We transformed *N. attenuata*, silencing its *RCA* and *RUB* to determine whether the reductions in photosynthetic capacity that commonly result translate into reductions in: 1) growth with and without intense intra-specific competition; and/or, 2) resistance to generalist herbivores.

Decreased expression of *Narca* and *Narub* was accompanied by a decrease in RuBPCase activity and a reduction in net photosynthetic rate at the whole plant level. At optimum CO_2 concentrations (400 μmol mol^{-1}) and different light intensities, photosynthesis is decreased by 50%. However, under different CO_2 concentrations and saturated light intensity, the photosynthesis rate decreased by 20% compared to EV-transformed plants. Eckardt et al. (1997) demonstrated that *RCA* mutants of *Arabidopsis thaliana* with 30-40% reductions in *RCA* content were significantly diminished in their photosynthetic capacity and growth; this effect was even more pronounced under high light conditions (600 μmol/m^{-2} s^{-1}). These results are consistent with our observations and demonstrate that increases in light intensity cannot improve the performance of transformed plants; however, transformed plants can recover up to 30% of their photosynthetic capacity when the CO_2 supply is increased. This result indicates the impaired activity of RuBPCase after silencing *RCA* and *RUB* in *N. attenuata*. At the rosette stage, the photosynthesis rates of RCA-and RUB-silenced plants are similar to each other; however, at the whole plant level, photosynthesis is reduced by 50% in RCA-silenced plants and 25% in RUB-silenced plants. Stalk length in RCA-silenced plants is decreased as photosynthesis do; though *RUB*-silenced plants grow slowly compared to EV-transformed plants, they tend to catch up to EV-transformed plants. Whole-plant measurements of photosynthesis rates and stalk lengths suggest that the production of RuBPCase is more than is required to maintain plant growth.

The performance of *S. littoralis* improved on *RCA*-silenced plants. The improved herbivore performance on *RCA*-silenced plants confirms the measures of defense metabolites

namely, TPIs and DTGs, in these plants. DTGs and TPIs are well known for their anti-herbivore activity (Zavala et al., 2004; Jassbi et al., 2007; Mitra et al., 2007: in review). Therefore decreased accumulation of DTG and TPI in irRCA plants positively influences *S. littoralis*'s growth. The unaltered body mass of *S. littoralis* on asRUB plants is also consistent with the unaltered amount of DTG and TPI activity. The seeming paradox between the accumulation of defense metabolites and resistance traits in *RCA*- and *RUB*-silenced plants led us to scrutinize their performance in defense signaling. We found that though both have a similar jasmonic acid (JA) burst, RCA-silenced plants are significantly impaired in jasmonate-Ile/Leu accumulation 45 minutes after induction with W+OS compared to EV plants. JA-Ile is the main jasmonate amino-acid conjugate known for defense signaling (Wang et al., 2007). RCA-silenced plants complemented with external Ile were unable to increase their JA-Ile/Leu levels. The transcript accumulation of *JAR*4/6, the key enzyme for JA-Ile conjugation (Wang et al., 2007) in irRCA plants, is similar to that in EV-transformed plants. The results from the complementation experiments and transcription profiles of JAR4 suggest that JA-Ile levels in RCA-silenced plants are influenced neither by the Ile pool nor by the lower conjugation activity of JA and Ile. High levels of dietary protein are known to be able to negate the effects of TPI ingestion on the growth of larvae (Johnson et al., 1996). In the present study, levels of RuBPCase, the main dietary protein in leaves, were about 1.5 times higher in irRCA plants than in EV-transformed plants, whereas they were1.5 times lower in asRUB plants than in EV-transformed plants. It is known that *RCA* silencing delays the turnover of RuBPCase and increases its proportional representation in the total protein pool of rice and cultivated tobacco (He et al., 1997; Jin et al., 2006) . Therefore, the increased level of RuBPCase in *RCA*-silenced plants gives additional nutrition to *S. littoralis* larvae, allowing them to gain more body mass compared to larvae that fed on *RUB*- and EV-transformed plants.

According to the carbon-nutrient balance hypothesis (Coley et al., 1985), secondary metabolism is directed towards carbon-rich metabolites in nitrogen-limited plants and vice versa. Matt et al. (2002) demonstrated that decreasing photosynthetic capacity by down-regulating RuBPCase transcripts in cultivated tobacco strongly decreased the accumulation of carbon-rich secondary metabolites, namely chlorogenic acid and rutin. DTG is also a C-rich metabolite and decreased photosynthesis likely affects DTG accumulation. However, asRUB plants have similar rates of photosynthesis as irRCA plants at the rosette stage and have similar levels of DTG accumulation compared to EV-transformed plants. This result suggests that resource allocation is influenced by whole plant photosynthesis. Plants gain resources via

photosynthesis and allocate them to growth, reproduction, and defense. Zangerl and Berenbaum, 1992 tried to address the question of how resource should be allocated to defense and other sinks, but they conclude that the field of resource allocation is still in its infancy. However, investing resources in both reproduction and anti-herbivore defense is costly especially in the absence of effective herbivores (Rausher et al., 1993; Saulnier and Reekie, 1995). Therefore, the resources gained and the allocation strategies used varied according to the ecological situation and the plant species involved. (Coley, 1998)nerally, plant growth is negatively related to plant defense (Coley et al., 1985; Coley, 1998). In our study, transformed plants are on the one hand slow growing and on the other hand less resistant to herbivores. These observations raise the question, how do *N. attenuata* plants allocate their carbon resources? According to Smith and Stitt (2007), newly assimilated carbon is allocated to the most immediate demand and also stored for the anticipated demand. Our results suggest that the resource allocation in *N. attenuata* probably leans more towards the "anticipated" demand than "immediate" demand. This hypothesis can explain why the stalk length of RUB-silenced plants catches up to that of EV-transformed plant at later growth stages.

N. attenuata's photosynthetic capacity is likely larger than that required to maintain defense responses. Therefore, *N. attenuata* plants can recover their growth at a late growth phase and maintain their defensive traits even after a 50% decrease of the main photosynthetic enzyme RuBPCase in levels of transcripts and proteins. Moreover, the combination of results from complementation experiments, transcription profiles of *JAR*4/6 and whole plant photosynthesis suggest that JA-Ile levels in *RCA*-silenced plants are influenced by carbon metabolism than Ile pool and lower conjugation activity of JA with Ile. In *N. attenuata* plants, pretreatment with glucose can increase JA-Ile accumulation in wild-type plants; levels of JA-Ile/Leu in *RCA*-deficient plants pretreated with glucose are similar to those in EV-transformed plants (unpublished data) 45 minutes after W+OS treatment. Therefore, glucose presumably plays an important role in JA-Ile accumulation in transformed plants: either the glucose signaling or glucose metabolism influences JA-Ile/Leu accumulation in *RCA*-silenced plants. However, how this occurs required further experiments. We conclude that *N. attenuata*'s total photosynthetic capacity influences resource allocation, which in tern affects growth, defense metabolite production, and resistance to herbivores.

MATERIALS AND METHODS

Plant Growth Conditions

Seeds of a *Nicotiana attenuata* inbred line were smoke-germinated on GB medium (Krugel et al., 2002). Ten days after germination, seedlings were planted in soil into Teku pots (Waalwijk, the Netherlands) and after 12 additional days, transferred to 1L (singly-grown) or 2L (competition-grown) pots with a peat-based substrate (Klasmann Tonsubstrat, Geeste-Groß Hesepe, Germany). Plants were grown in the glasshouse of the Max Planck Institute for Chemical Ecology (Jena, Germany) at 24-26°C (16h light; supplemental lighting by Philips Sun-T Agro (http://www.nam.lighting.philips.com/) 400 and 600 W sodium lights ; 55% humidity). Four- to five-week-old plants were used for all experiments except biomass measures.

Silencing *RCA* and *RUB* in *N. attenuata*

Vector Construction and *Agrobacterium*-mediated Transformation

A 268bp inverted-repeat fragment of the cDNA sequence of *Narca* was inserted into the pREC5 transformation vector and a 316 bp of the cDNA sequence of *Narub* was inserted into the pREC2 transformation vector in antisense orientation (Bubner et al., 2006). This vector along with the *Narca* and *Narub* insert was transformed into *N. attenuata* WT plants using *Agrobacterium*-mediated transformation (Krugel et al., 2002). The presence of the hygromycin resistance gene (*hptII*) in the transformation vector allowed us to identify hygromycin-resistant transformants (Krugel et al., 2002). The number of insertions was determined by Southern hybridization of genomic DNA using a PCR fragment of the *hptII* gene as a probe. Single-insertion lines of irRCAand asRUB plants were identified and used in all subsequent experiments with the same WT generation transformed with an empty vector (EV).

Southern Blot Analysis and Quantitative RT-PCR

DNA was extracted from the fully expanded leaves of *N. attenuata* plants using the cetyltrimethylammonium bromide (CTAB) method (Rogers and Bendich, 1985) with slight modifications. 10 µg of the DNA samples were digested with different restriction enzymes, size-fractionated in a 0.8% (w/v) agarose gel, and Southern blotted (Brown , 1997) onto a nylon membrane (GeneScreen Plus, Perkin Elmer, Rodgau-Jügesheim, Germany;

http://las.perkinelmer.com/). Fragments of *Narca* (forward primer: 5'-GAAGCTCCTTGAGTATGGTAACATG-3'; reverse primer: 5'-GGCAGCACTTGGAGATGCAAATG-3'), *Narub* (forward primer: 5'-CGGACGTTGTCGAATCCAATG-3'; reverse primer: 5'-CGAGACACTCTCATACCTTCC-3') and of *hptII* (forward primer: 5'-CGTCTGTCGAGAAGTTTCTG-3'; reverse primer: 3'- CCGGATCGGACGATTGCG- 5'), respectively, were amplified by PCR and used as probes for Southern hybridization. The probes were labeled with a-^{32}P (Rediprime™ II DNA labeling system, Amersham Biosciences, Freiburg, Germany; http://www.amersham.com/).

To analyze *Narca, Narub, and NaJAR4/6* expression, we extracted total RNA with TRI reagent protocol (Sigma, Taufkirchen, Germany; http://www.sigmaaldrich.com/). cDNA was synthesized from 2μg RNA using superscript II™ reverse transcriptase (Invitrogen; http://www.invitrogen.com/). Quantitative RT-PCR was conducted using a gene-specific primer pair (*Narca*: forward primer: 5'- AGAACGCCAGGGTCCCTATT -3', reverse primer: 5'- CGACCATCACGGATAAGAGGA - 3'; *Narub:* forward primer: 5'-CGAGACACTCTCATACCTT-3'; reverse primer: 5'-GTAGTACCCTGGTGACTT-3'); *NaJAR*4: forward primer: 5'- ATGCCAGTCGGTCTAACTGAA -3', reverse primer: 5'-TGCCATTGTGGAATCCTTTTAT - 3'; *NaJAR*6: forward primer: 5'-TGGAGTAAACGTTAACCCGAAA -3', reverse primer: 5'-AGAATTTGCTTGCTCAATGCCA - 3') . The relative gene expression was calculated using an *actin* primer which served as endogenous control gene.

CO_2-exchange and Plant Growth

In the glasshouse four-week-old plants were used for CO_2-exchange measurement. Net photosynthetic rates and intercellular CO_2 concentrations were measured on RCA-, RUB-, and EV-transformed plants under saturating light (1200μmol m^{-2} s^{-1}) using Li-Cor 6400 Portable Photosynthesis System (LiCor Biosciences, Lincoln, NE, USA; http://www.licor.com/). Net photosynthesis was measured in at least five replicate plants each at six different CO_2 concentrations, namely, 650,600, 400, 300, 200, 100, and 0 μmol mol^{-1}. Photosynthesis was also measured in the different leaf positions, namely, S2 (position 1), S1 (position 2), and +2 (position3) at optimum (400 μmol mol^{-1}) CO2 concentration and saturated light intensity (1200 μmol m^{-2} s^{-1}). The slope of a regression of photosynthetic rate against C_i provides an *in vivo* measure of RuPBCase activity (Farquhar et al., 1980) and the slope of the A/Ci curve of EV plants is ~1.2 times greater than that of the irRCA plants,

which clearly demonstrates that silencing *RCA* reduced RuBPCase activity; reduced RuBPCase activity in turn decreased net photosynthesis. A light responsive curve of photosynthesis was generated in at least five replicate plants each at optimum CO_2 concentration (400 μmol mol^{-1}) and six different light intensities, namely, 0, 200, 500, 1200, 1500, and 2000 μmol m^{-2} s^{-1}. Stalk length was measured in four-week-old plants for seven consecutive days to evaluate growth.

Analysis of Diterpene Glycosides and Trypsin Protease Inhibitors

Leaf tissue (~100mg) was sampled to analyze levels of diterpene glycoside and trypsin proteinase inhibitors. The accumulation of DTGs was analyzed by high-performance liquid chromatography as previously described (Halitschke et al., 2003). Trypsin proteinase inhibitor (TPI) activity was analyzed by the radial diffusion assay described by (van Dam et al., 2001).

Quantification of JA and JA-Ile

About 200 mg of harvested leaf tissue from each line were analyzed for JA and JA-Ile level by 1200L LC/MS system (Varian, Palo Alto, CA, USA; http://www.varianinc.com/). Samples were extracted using the method described by Wang et al. (2007).

Performance generalist herbivore *S. littoralis*

Freshly hatched *S. littoralis* larvae were grown on artificial diet for 6 days and then put on the transformed plants. After feeding for 2 days, larvae were weighed every second day for 12 days.

Statistical Analysis

Data were analyzed with StatView (Abacus Concepts, Inc., Berkeley, CA, USA; http://abacus-concepts.com/). Experiments in the greenhouse were analyzed by the analysis of variance (ANOVA) for singly-grown plants and by paired t-test for competition-grown plants.

ACKNOWLEDGEMENTS

We thank K. Gase and T. Hahn for vector construction and DNA sequencing; S. Kutschbach, A. Wissgott, J. Wurlitzer and T. Guse for generating transformed plants; E.

Rothe and M. Schöttner for LC-MS analysis; T. Krügel, A. Weber, and A. Schüenzel for growing the plants and E. Wheeler for editorial assistance.

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4. Discussion

In their native environments, plants use numerous and dynamic defense strategies against herbivores and pathogens. Previous research, using a detailed microarray and secondary metabolite analysis, demonstrated that the two most abundant fatty-acid amino acid conjugates (FACs) in *Manduca sexta* oral secretions (OS) can account for all measured direct and indirect defenses, the endogenous jasmonic acid burst that elicits them, and 65-86% of the induced transcriptional changes elicited by attack on *N. attenuata* leaves (Halitschke et al., 2001; Roda et al., 2004). The unbiased querying of the transcriptomes of plants attacked by herbivores has revealed unexpected functions of genes which would have been difficult to predict given their established biochemical function. A way to examine gene function is to study plants lacking the expression of respective genes. The Arabidopsis research community permanently generates mutants exhibiting single or multiple defects in every aspect of plant metabolism in order to explain gene function. Transgenic technology also offers the possibility to reduce endogenous gene expression by antisense or inverted repeat expression of the respective genes. In *N. attenuata*, plants are being transformed with antisense and inverted-repeat constructs of genes with putative roles in plant defense to study these genes' function in defense (Halitschke et al., 2001; Steppuhn et al., 2004; Zavala et al., 2004). However, how changes in the transcriptome translate into changes in the proteome and metabolome that eventually account for the phenotype of increased resistance is unknown. We are beginning to understand how plants solve individual components of this multidimensional challenge. Despite the recognized importance of whole-organismic gene function, biologists are better equipped to empirically explore a gene's biochemical function than its function at an organismic level, due primarily to the availability of excellent biochemical expression systems. The paucity of whole-organism expression systems and our incomplete understanding of the natural history of many molecular model organisms have hampered our ability to understand gene function at the level of the organism. Therefore, we attempt to reveal not only the changes in plant's proteome after herbivory but also the function of herbivore-regulated atypical proteins at the organismic level.

The present dissertation provides substantial evidence that to cope with its herbivorous enemies *N. attenuata* reorganized the accumulation of its proteins, which are involved in different functions (Manuscript I). We want to understand why these proteins are regulated after herbivore attack on the mechanistic and functional level. Mechanistically, we are interested in questions such as, which factor of *M. sexta*'s oral secretions is responsible for the

large-scale proteomic changes? Are proteomic and transcriptomic changes correlated with each other? Functionally, we are interested in questions such as, Do these changes in the proteome indicate the manipulation of plant metabolism by the herbivore and as a result increase plant resistance? How do herbivores overcome resistance? In general, an induced plant is known to decrease its photosynthesis rate while increasing the production and accumulation of defense-related compounds (Walling, 2000; Hermsmeier et al., 2001; Kessler and Baldwin, 2002; Hahlbrock et al., 2003). It has been shown that herbivore-induced reductions in photosynthetic rates are greater than the amount of canopy area removed by the herbivore (Zangerl et al., 2002). Therefore, we have extended our research to understand how the large decrease in photosynthesis rate influences herbivory (Manuscript III) by directly manipulating the photosynthetic capacity of *N. attenuata* plants.

The discussion is organized in four sections: 1) Proteomics of plant-herbivore interaction 2) Correlation between proteomic and transcriptomic changes 3) Querying the proteome for how plants cope with herbivore attack, and 4) photosynthesis and plant defense.

Proteomics of plant-herbivore interaction

High-throughput transcriptional analysis of the interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *N. attenuata* identified a large-scale transcriptional reconfiguration, which caused decreases in photosynthesis-related processes and increases in defense-related processes (Walling, 2000; Halitschke et al., 2001; Halitschke et al., 2003; Hui et al., 2003). Here we extend the analysis to the level of the proteome of *N. attenuata*-*M. sexta* interaction and treated plants by following ways: treatment of caterpillar feeding, wounds treated with water, four synthetic FACs, and FAC-free OS. Synthetic FACs account for 19 of the 32 proteins elicited by W+OS treatment. Moreover, excluding FACs by ion-exchange chromatography removes the regulation of 8 proteins and treatment with FAC-free OS elicits only 1 protein which was not elicited by treatment with synthetic FACs. It is interesting to note that the response of certain proteins can not be attributed to the presence of FACs. We identified 7 different functional photosynthesis-related proteins (out of 20 protein spots), which accumulate differentially in herbivore-induced leaf tissues. We excluded RuBPCase from the analysis because its transcripts are known to degrade in response to herbivore and pathogen attack (Hermsmeier et al., 2001; Hahlbrock et al., 2003; Hui et al., 2003). A substantial change was observed in the accumulation of the

protein RuBPCase activase (RCA). RCA is a molecular chaperon and known to modulate the activity of RuBPCase, the main photosynthetic protein. Chaperonins assist in folding a wide range of structurally and functionally unrelated proteins, and it is possible that RCA interacts with proteins other than RuBPCase when plants are attacked or that the RCA subunit content and its composition contribute to optimizing plant performance during attack. Plants with decreased levels of RuBPCase activase protein had reduced photosynthetic rates and less RuBPCase activity as well as less biomass, responses consistent with those of herbivore-attacked plants. Therefore, a detailed characterization of RCA-deficient plants was also carried out (Manuscript III). Of the 28 primary metabolism proteins that were differentially regulated, 8 are involved in amino acid metabolism and were up-regulated in response to OS elicitation: TD, S-adenosylmethionine synthetase, O-acetylserine thiol lyase, spermidine synthase, Gln synthetase, 3-dehydroquinate dehydratase, homo-Cys-S-methyltransferase, and hydroxymethyltransferase. Seven out of 8 proteins involved in the production of secondary metabolites showed increased accumulation levels at least one time point after OS elicitation.

For our analysis of 90 herbivore-elicited protein spots in over 84 2-DE gels, we not only developed the techniques for extracting, separating, and analyzing complex protein mixtures reproducibly, but we also characterized proteomic responses to elicitation and learned that the larval elicitors responsible for the transcriptional response account for a majority of the specific changes in the proteome. FACs, plant defense elicitors that are synthesized in the larval midgut, elicits defense responses in the plant that appear to benefit the plant. In general, proteins that increased were involved in primary metabolism, defense, and transcriptional and translational regulation; those that decreased were involved in photosynthesis. However, other elicitors are produced by the plant during caterpillar attack and some of these may benefit the caterpillar. Recent work from our group has shown that treatment of wounds with OS and larval attack dramatically increases methanol (MeOH) emissions from attacked plants. This MeOH release, which is elicited not by the FACs in OS but also by the high pH of OS (pH 9.3), is associated with increases in transcripts and the activity of leaf PME and decreases in the degree of pectin methylation (von Dahl et al., 2006). This analysis revealed that FACs play a major role in organizing not only transcriptional responses but also proteomic responses. This dual role is not simply a result of an overlap of the transcriptional and proteomic responses to FACs because the responses of the transcriptome and proteome are clearly very different.

Correlation between proteomic and transcriptomic changes

It is evident from several studies that the proteomic analysis of a plant's response does not necessarily match the transcriptional analysis of the same response. In the well-studied eukaryotic system of yeast, similar mRNA expression levels were accompanied by a wide range of protein abundance levels and vice versa (Gygi et al., 1999). Chilling rice plants changes the abundance of several mRNA transcripts; such changes are not reflected in the changes in their corresponding proteins (Yan et al., 2006). In general, the mRNA-protein association depends on three factors: (i) mRNA expression level, (ii) rate of protein synthesis per mRNA, and (iii) rate of protein degradation per protein (Greenbaum et al., 2002). Changes in any one factor can cause a discrepancy in the association between mRNA and protein accumulation. Though protein synthesis and its turnover are highly regulated processes, stress conditions are known to reorganize protein populations (Huang et al., 2005; Morgenthal et al., 2006; Nie et al., 2006; Plaxton and Podesta, 2006; Renaut et al., 2006). Protein levels of a cell can be controlled transcriptionally or translationally, independent of mRNA and protein concentrations (Greenbaum et al., 2002). Moreover, diversity and abundance in the proteolytic machinery add further complexity to the comparison between mRNA and ever-changing protein populations (Smalle and Vierstra, 2004; Kwon et al., 2006). We hope to provide a glimpse of the correlation between proteomic and transcriptomic changes.

Different peptide sequences obtained from protein spots were used to design primers to compare transcript and protein accumulation patterns by a semiquantitative reverse-transcription PCR approach. We compared accumulation levels in leaves of 17 gene transcripts with those of their corresponding proteins at five time points (6h, 12h, 30h, 48h, and 72h) after elicitation with different insect-derived signals. The selected candidates were trypsin protease inhibitors (*tpi*), threonin deaminase (*td*), phosphoglycerate mutase (*pgm*), S-adenosylmethioninesynthetase (*sams*), pectin methyl esterase (*pme*), glyceraldehyde-3-P dehydrogenase (*gpdh*), mitochondrial formate dehydrogenase (*fdh*), vacuolar ATPase(*atpe*), glycine-rich RNA binding protein (*grp*), transketolase (*tk*), RuBPCase activase (*rca*), chaperonin (*cpn*), Mg-protoporphyrin IX chelatase (*mgpc*), transcription elongation factor (*tef*), oxygen evolving protein (*oep*), glutamine synthase (*gmps*), and aldolase (*ald*). Six genes showed transcript patterns that correlated strongly with the patterns of protein abundance in the time series analysis. The mRNA and protein levels of the remaining 10 proteins were differently regulated when compared in the time series analysis after OS elicitation.

Comparison of protein and mRNA accumulation after different elicitation treatments identified seven members, namely, *tpi, td, pgm, sams, fdh, gpdh,* and *atpe,* which shared similar patterns.

Querying the proteome for how plants cope with herbivore attack

Analysis of the molecular interactions between the specialist herbivore *M. sexta* and its natural host, *Nicotiana attenuata*, has identified several combinations of direct and indirect defenses which are demonstrably important for the plant's Darwinian fitness, especially in natural environments (Kessler et al., 2004; Steppuhn et al., 2004; Zavala et al., 2004). However, our proteomic analysis of leaves attacked by *M. sexta* revealed that many proteins are differentially regulated during the interaction; their role, however, remains a mystery, as their biochemical function, inferred from their sequence similarities, provides no known association with potential defense responses. To find out the functions of atypical herbivore-responsive proteins, we conducted an organismic-level analysis of 7 differentially regulated proteins in the *M. sexta-N. attenuata* interaction. To be selected, genes had to (i) have well-established kinetics of transcript and protein accumulation in the *N. attenuata-M. sexta* interaction, (ii) have no established association with defense in the *N. attenuata-M. sexta* interaction, and (iii) be involved in different processes in the plant. We used a virus-induced gene silencing (VIGS) system optimized for *N. attenuata* (Saedler and Baldwin, 2004) to reduce the accumulation of the transcripts of the regulated proteins and examined the progress of the plant-insect interaction in currencies which reflect both sides of the interaction, namely, the plant's and the herbivore's. Four up-regulated proteins – namely, vacuolar ATPase (ATPE), which is involved in ion and metabolite transport and cell enlargement (Padmanaban et al., 2004); cinnamyl alcohol dehydrogenase (CAD), which is involved in the production of lignin monomers; chaperonin (CPN), which is involved in the association of RuBPCase subunits (Cloney et al., 1992); and transketolase (TK), which is involved in Calvin cycle (Henkes et al., 2001)– and three down-regulated proteins – namely, glyceraldehyde-3-phosphate dehydrogenase (GPDH), which is involved in glucose metabolism; glutamine synthase (GMPS), which is involved in the photorespiratory carbon cycle, where it re-assimilates ammonia (Hausler et al., 1996); and oxygen-evolving protein (OEP), which is involved in the light reaction of photosynthesis – were used in the gene-silencing experiments.

The expression of candidate proteins was silenced by virus-induced gene silencing (VIGS) and categorized according to the proteins' influence on the two main dependent variables of this insect- plant interaction: photosynthetic performance (reduced or unchanged compared to EV plants) and *M. sexta* performance (reduced, unchanged, or more larval mass compared to larvae that fed on EV plants). It is known that organism size greatly affects the rate of cellular metabolism (Schmidt-Nielsen, 1984); moreover, the dependence of a biological variable on body mass is typically characterized by allometric scaling, which may be tissue-specific (West et al., 1997). If changes in secondary metabolism are coupled with changes in specific leaf area, then metabolism may not affect concentration (Hamilton et al., 2001). Moreover, concentration of a secondary metabolite depends not only on the synthesis of that particular compound but also on the synthesis of other plant constituents (Koricheva, 1999). Therefore, we expressed the metabolite production of silenced plants in a currency of whole-plant pools as well as concentrations. Our results suggested that after herbivory, plant proteins are selectively up- and down-regulated in an adaptive manner. Apart from their known biochemical functions, candidate proteins play direct and/or indirect roles in plant herbivore interaction by influencing the metabolic framework in both plants and insects. We found that silencing some of the candidate proteins affects caterpillar performance more than silencing the expression of known defense proteins. From multiple regression analyses, we infer that herbivore performance was most affected by DTG concentrations; however, that variation in DTG could not account for all the variation in larval performance suggests that other unknown defenses and/or synergistic interactions among metabolites remain to be discovered. Phenolics are generally thought to function as defenses, so it was surprising to find a strong positive correlation between rutin concentrations and larval performance. However, our result is consistent with the findings from (Deboer and Hanson, 1987). In addition, the analysis identified new controls over the regulation of growth and defense in *N. attenuata*. For example, the control of N allocation over inducible and constitutive nicotine and TPI production in *Nagmps*-silenced plants and the significant increase in growth of *Nacad*-silenced plants despite unchanged photosynthetic rates illustrate a new mode of metabolite allocation. Larval performance on *Nacpn*-silenced plants and the lack of a correlation between TPI and gut protease accumulation raise important questions about how the chaperonic activity of *Nacpn* contributes to DTG production and influences other defense traits.

We used an unbiased analysis of insect midgut proteome to determine if *M. sexta* larvae adjusted their digestive physiologies to cope with variation in host nutritional quality.

A comparative analysis of the 2DE profile of larval midgut proteins of larvae that fed on EV, *Nacad-, Nacpn-*, and *Nagmps*-silenced plants was done because the larvae reared on these plants had body masses that were similar (*Nacad*), more (*Nacpn*), and less (*Nagmps*) than the mass of larvae reared on EV. The analysis suggested that silencing different candidate genes in host plants affects *M. sexta*'s mid-gut proteome in distinct ways. The low body mass of pTV*gmps*-fed larvae indicated suboptimal nutritive conditions, which correlated with the largest number of up-regulated proteins. The high body mass of pTV*cpn*-fed larvae indicated optimal nutritive conditions compared to pTV*gmps*-fed larvae and correlated with the smallest number of up-regulated proteins. Opposite trends were found in the down-regulated protein spots: the largest numbers of proteins were down-regulated in pTV*cpn*-fed caterpillars and the smallest in pTV*gmps*-fed caterpillars. Therefore, the up-regulation of a number of defense-related proteins may be necessary to adapt to difficult diets.

Photosynthesis and plant defense

In the study of *N. attenuata-M. sexta* interactions, a substantial change was observed in the accumulation of RuBPCase activase (RCA) and RuBPCase at the transcript and protein level after wounding (W) and applying *M. sexta* regurgitate (OS) to wounds, a process which elicits *M. sexta*-specific responses (Halitschke et al., 2001; Roda et al., 2004; Giri et al., 2006). The key role of *RCA* is to modulate the activity of RUBPCase, the major photosynthetic protein, by removing the inhibitory sugar phosphates from the active site of the enzyme, whether or not the enzyme is carbamylated (Portis, 2003). Herbivore attack reduces a plant's photosynthetic capacity more than can be attributed to the removal of canopy area by the herbivore (Zangerl et al., 2002). Under such conditions plants must grow rapidly to compete and simultaneously maintain the defenses necessary to survive in environments with herbivores (Herms and Mattson, 1992). The dramatic up-regulation of defense metabolite production likely requires metabolic adjustments in plant growth and reproduction (Halitschke et al., 2003; Reymond et al., 2004; Ralph et al., 2006). Though it is believed that when attacked by herbivores, a plant appears to choose between growth and defense responses (Herms and Mattson, 1992), (Smith and Stitt, 2007) suggested that the interplay between resistance and growth is not in a straight competition when reduction in growth is due to direct carbon limitation. Therefore, manipulating photosynthesis by silencing the expression of *RCA* and *RUB* in *N. attenuata* can reveal how herbivore resistance is influenced by reducing carbon resources. Decreased expression of *Narca* and *Narub* was

accompanied by decreased RuBPCase activity, a 50% reduction in net photosynthetic rates at the optimum CO_2 concentration (400 μmol mol^{-1}), and different light intensity. The growth of RCA- and RUB-silenced plants is consistent with the rate of photosynthesis; though *RUB*-silenced plants grow slowly compared to the EV-transformed plants, there is a tendency to eventually catch up the height of EV-transformed plants. This observation suggests that the production of RuBPCase is more than is required to maintain plant growth. Generalist (*Spodoptera littoralis*) herbivores grew faster when fed on *RCA*-silenced plants, which is consistent with the decreased accumulation of trypsin protease inhibitors (TPIs) and diterpene glycosides (DTGs). In contrast, the body masses of *S. littoralis* on RuBPCase-silenced plants are similar to those of *S. littoralis* on EV plants, which is consistent with unaltered production of TPIs and DTGs. In the present study, levels of RuBPCase, the main dietary protein in leaves, were about 1.5 times higher in irRCA plants than in EV plants, whereas levels were about 1.5 times lower in asRUB plants than in EV-transformed plants. *RCA* silencing is known to delay the turnover of RuBPCase and increases its proportional representation in the total protein pool of rice and cultivated tobacco (He et al., 1997; Jin et al., 2006) . Therefore, the increased level of RuBPCase in *RCA*-silenced plants adds nutrition to *S. littoralis* larvae, allowing them to gain more body mass compared to *S. littoralis* larvae that fed on RUB- and EV-transformed plants. The seeming paradox between the accumulation of defense metabolites and resistance traits in RCA- and RUB-silenced plants leads us to scrutinize their performance in defense signaling. We found that though both of them have a similar jasmonic acid (JA) burst compared to EV plants, RCA-silenced plants are significantly impaired in jasmonate-Ile/Leu accumulation 45 minutes after induction with W+OS. RCA-silenced plants complemented with external Ile were also unable to increase their JA-Ile/Leu levels, as were EV plants. The transcript accumulation of *JAR*4 and *JAR*6, the key enzyme for JA-Ile conjugation in irRCA plants, is similar and increased respectively to that in EV-transformed plants. The combination of results from the complementation experiments and transcription profiles of JAR4/6 suggested that JA-Ile levels in RCA-silenced plants are influenced neither by the Ile pool nor by the lower conjugation activity of JA and Ile. In *N. attenuata* plants, pretreatment with glucose can increase JA-Ile accumulation in wild-type plants; levels of JA-Ile/Leu in RCA-deficient plants pretreated with glucose are similar to those in EV-transformed plants (unpublished data) 45 minutes after W+OS treatment. Therefore, glucose presumably plays an important role in JA-Ile accumulation in transformed plants: either the glucose signaling or glucose metabolism influences JA-Ile/Leu accumulation in RCA-silenced

plants. However, how glucose influences JA-Ile/Leu pool in *N. attenuata* plants is still unknown and requires further experiments.

We conclude that silencing the genes of proteins differentially regulated during a plant-herbivore interaction and querying each player in the interaction will reveal novel functions of genes at an organismic level.

5. Summary

Since plants are largely immobile, they have evolved a large degree of physiological plasticity to cope with fluctuating environments. Plastic responses to herbivore attack involve transcriptional reorganizations and increases in hormone concentrations, enzyme activities, and secondary metabolite levels. Plants respond polygenetically to herbivores with different feeding habits by large transcriptomic reorganization upon herbivory. A comparative proteomic analysis examined whether the large-scale changes in transcriptome also translated into large-scale changes in the proteome.

Experiments were conducted with a native system: a wild species of tobacco (*Nicotiana attenuata*) from the Great Basin Desert which served as host for chewing larvae of *Manduca sexta*, *Spodoptera exigua*, and *Heliothis virescense*, and the mesophyll-sucking *Tupiocoris notatus*. Phloem-feeding aphids (*Myzus nicotinae*), though not found on *N. attenuata* in nature, easily colonized greenhouse plants. All these herbivores are major pests on either Solanaceous or non-Solanaceous crops. Detailed research on wound- and herbivore-induced responses in *N. attenuata* using ecological, analytical, and molecular approaches (Baldwin and Peterson 1999, Baldwin 2001; Voelckel and Baldwin 2004) preceded the work that is summarized in three manuscripts.

We identified proteins in *N. attenuata* whose expression substantially changes in response to different elicitors, which mimic the feeding of *M. sexta* larvae by 2-dimensional gel electrophoresis and MALDI-TOF, LC-MS-MS analysis (I). We analyzed the function of 7 atypical herbivore-responsive proteins by silencing their expression in *N. attenuata* plants via virus-induced gene silencing to reveal their influence at organismic level (II). In addition, we evaluated *N. attenuata* plants with decreased levels of photosynthesis for their resistance to herbivores (III).

I. Proteomic studies confirm that herbivore attack reorganized plants' protein profiles by suppressing and enhancing wound-induced proteins. The proteomic signatures elicited by *M. sexta*'s oral secretions (OS) correlate with the profile of elicited fatty acid amino acid conjugates (FACs). Therefore, as in the transcriptional response, FACs are the main elicitor in OS responsible for proteomic reorganization. Phenolic extracts yielded approximately 600 protein spots, many of which were altered by elicitation, whereas nuclear protein fractions yielded approximately 100 spots, most of which were unchanged by elicitation.

Reproducible elicitor-induced changes in 90 spots were characterized. In general, proteins that increased were involved in primary metabolism, defense, and transcriptional and translational regulation; those that decreased were involved in photosynthesis. A semiquantitative reverse-transcription PCR approach based on peptide sequences was used to compare transcript and protein accumulation patterns for 17 candidate proteins. In six cases the patterns of elicited transcript accumulation were consistent with those of elicited protein accumulation. Functional analysis of one of the identified proteins involved in photosynthesis, RuBPCase activase, was accomplished by virus-induced gene silencing. Plants with decreased levels of RuBPCase activase protein had reduced photosynthetic rates and RuBPCase activity, and less biomass, responses consistent with those of herbivore-attacked plants.

II. We use an unbiased means of evaluating the function of these reconfigured proteins for the plant-herbivore interaction. Their function was tested by silencing genes coding for 7 herbivore-induced proteins not known to play obvious defensive roles in the *Nicotiana attenuata-Manduca sexta* interaction and by analyzing the performance of plants and insects traits known to mediate defense responses. Our "ask the proteome" approach revealed that silencing genes of up-regulated proteins in *N. attenuata* tended to benefit larval performance, while silencing genes of down-regulated proteins tended to diminish it: these results are consistent with expectations that herbivory-induced regulation reflects the proteins' function in attacked plants. However, the discrepancy between variation in known defense metabolites and larval performance suggests other metabolites are more important. An unbiased analysis of insect midgut proteome was carried out to determine if *M. sexta* larvae adjusted their digestive physiologies to cope with variation in host nutritional quality. A comparative analysis of the 2-DE profile of larval midgut proteins suggested that silencing different candidate genes in host plants affects *M. sexta*'s mid-gut proteome in distinct ways. The low body mass of pTV*gmps*-fed larvae indicated suboptimal nutritive conditions, which correlated with the largest number of up-regulated proteins, whereas the high body mass of pTV*cpn*-fed larvae indicated optimal nutritive conditions compared to pTV*gmps*-fed larvae, which correlated with the smallest number of up-regulated proteins. These observations suggested that silencing the genes of proteins

differentially regulated during a plant-herbivore interaction and querying each player in the interaction can reveal novel functions of genes at an organismic level.

III. Herbivore attack reduces the levels of photosynthetic proteins and a plant's photosynthetic capacity, whereas it increases the production and accumulation of defense-related metabolites. *N. attenuata* down-regulates RuBPCase activase and RuBPCase, two major photosynthetic enzymes, after herbivory to optimize its allocation of resources to growth, reproduction, and defense. Silencing these two genes in *N. attenuata* resulted in a large decrease in photosynthesis and growth. However, RCA-silenced plants are less resistant than RUB-silenced and EV-transformed plants. Herbivore performance on RCA- and RUB-silenced plants is consistent with their accumulation of jasmonate-Ile/Leu and defense metabolites. *N. attenuata*'s total photosynthetic capacity influences resource allocation, which in tern affects growth, defense metabolite production, and resistance against herbivores.

Characterizing more atypical herbivore-responsive proteins and comparatively analyzing metabolomic profile of transgenic plants may reveal new defense strategies of plants. In addition, an unbiased transcriptomic and proteomic analysis of herbivores fed on different transgenic lines may help researchers decipher how herbivores cope with plants' novel defense strategies.

6. Zusammenfassung

Da Pflanzen weitgehend unbeweglich sind, haben sie einen großen Grad and physiologischer Plastizität entwickelt, um mit wechselnden Umweltbedingungen zurecht zu kommen. Plastische Antworten gegenüber Angriffen von Pflanzenfressern schließen transkriptionelle Umorganisationen sowie Veränderungen in Hormonkonzentrationen, Enzymaktivitäten und Produkten des Sekundärstoffwechsels ein. Pflanzen antworten polygenetisch auf Pflanzenfresser mit unterschiedlichen Fraßgewohnheiten durch große Umorganisationen im Transkriptom. Im Rahmen einer vergleichenden Proteomanalyse wurde untersucht, ob sich die großen Veränderungen im Transkriptom auch im Proteom widerspiegeln.

Die Experimente wurden mit einem natürlichen System durchgeführt: eine wilde Tabakart (*Nicotiana attenuata*) aus der Great Basin Wüste in den USA, die als Wirt für kauende Larven von *Manduca sexta, Spodoptora exigua* und *Heliothis virescens* bzw. für mesophyllsaugende *Tupiocorus notatus* diente. Phloemsaugende Läuse (*Mycus nicotianae*) haben leicht Gewächshauspflanzen befallen, obwohl sie in der Natur nicht auf *N. attenuata* vorkommen. Alle diese Herbivoren sind wichtige Schädlinge entweder auf Feldfrüchten der Familie der Solanaceen bzw. Nicht-Solanaceen. Detaillierte Forschung zu wund- und Herbivor-induzierten Antworten in *N. attenuata* unter Verwendung ökologischer, analytischer und molekularen Methoden (Baldwin and Peterson 1999, Baldwin 2001, Voelckel and Baldwin 2004) sind der Arbeit, die in den drei Manuskripten zusammengefaßt ist, vorausgegangen.

Wir haben Proteine in *N. attenuata* durch 2-dimensionale Gelelektrophorese und MALDI-TOF, LC-MS-MS Analyse identifiziert, deren Expression sich als Antwort auf verschiedene Elicitoren, die den Fraß von *M. sexta* simulieren, grundlegend ändert (I). Wir haben die Funktion von sieben atypischen Proteinen, die auf Pflanzenfraß reagieren, durch Inaktivierung ihrer Expression mittels virus-induzierter Gen-Inaktivierung untersucht, um ihren Einfluß auf der Ebene des Organismus aufzudecken (II). Darüber hinaus haben wir *N. attenuata* Pflanzen mit verringerter Photosynthesehöhe im Hinblick auf ihre Resistenz gegenüber Herbivoren evaluiert.

I. Untersuchungen des Proteoms bestätigen, dass der Angriff von Herbivoren das Proteinmuster durch Unterdrückung oder Erhöhung wund-induzierter Proteine

umorganisiert. Das durch orale Sekrete (OS) von *Manduca sexta* elicitierte proteomische Muster korrelierte mit dem durch Fettsäurekonjugate (FAC) induzierten Profil. Daher sind, wie bei der Antwort auf Transkriptomebene, FACs im OS hauptsächlich für die Reorganisation des Proteoms verantwortlich. Phenolische Extrakte lieferten ungefähr 600 Proteinspots, viele von ihnen waren durch Elicitierung verändert, während die Fraktion der Kernproteine ungefähr 100 Spots lieferte, von denen die Mehrheit durch Elicitierung unverändert blieb. Reproduzierbare Elicitor-induzierte Veränderungen in 90 Spots wurden charakterisiert. Im Allgemeinen waren Proteine, die erhöht waren, in den Primärstoffwechsel, die Abwehr und transkritpionelle und translationelle Regulation involviert; die Proteine, die vermindert waren, waren an der Photosynthese beteiligt. Um die Transkript- und Proteinakkumulationsmuster von 17 Kandidatenproteinen zu untersuchen, wurde eine semiquantitative reverse Transkriptions-PCR auf der Grundlage der Peptidsequenzen durchgeführt. In sechs Fällen war das Muster der induzierten Transkriptanreicherung mit der induzierten Proteinanreicherung konsistent. Eine funktionelle Analyse von einem der identifizierten Proteine, das an der Photosynthese beteiligt ist, RuBPCase activase, erfolgte durch Virus-induzierte Gen Inaktivierung. Pflanzen mit verringerten Gehalten an RuBPCase activase Protein zeigten verminderte Photosyntheseraten und RuBPCase Aktivitäten, sowie verringerte Biomasse; Antworten, die mit denen eines Angriffs von Herbivoren übereinstimmen.

II. Wir haben einen unvoreingenommenen Ansatz verwendet, um die Funktion dieser rekonfigurierten Proteine für Pflanze-Herbivor-Interaktionen zu untersuchen. Ihre Funktion wurde untersucht, indem die Gene für 7 Herbivor-induzierte Proteine, die bislang nicht dafür bekannt waren, eine offensichtliche Rolle in der Abwehr in der *Nicotiana attenuata – Manduca sexta* Beziehung zu spielen, inaktiviert wurden. Außerdem wurden Merkmale von Pflanzen und Insekten untersucht, die bekanntermaßen an der Abwehr beteiligt sind. Unser „Frag' das Proteom"-Ansatz zeigte, dass die Inaktivierung von Genen von hochregulierten Proteinen in *N. attenuata* tendenziell die Larvenentwicklung positiv beeinflußte, während die Inaktivierung von Genen, deren Genprodukte herunterreguliert waren, tendenziell einen negativen Einfluß hatten: Diese Ergebnisse stimmen mit den Erwartungen

überein, dass Herbivorie-induzierte Regulation die Funktion der Proteine in der angegriffenen Pflanze widerspiegelt. Allerdings legt die Diskrepanz zwischen der Variation bekannter Abwehrstoffe und der Entwicklung der Larven nahe, dass andere Stoffwechselprodukte wichtiger sind. Eine unvoreingenommene Analyse des Proteoms des Insektenmitteldarms wurde durchgeführt, um zu bestimmen, ob *M. sexta* Larven ihre Verdauungsphysiologien anpassen, um mit den Schwankungen in der ernährungsphysiologischen Qualität ihrer Wirte zu Recht zu kommen. Eine vergleichende Analyse von 2 DE-Profilen von Proteinen des Larvenmitteldarms legt nahe, dass die Inaktivierung verschiedener Kandidatengene in Wirtspflanzen das Mitteldarmproteom von *M. sexta* unterschiedlich beeinflusst. Die geringe Körpermasse von pTV*gmps*-gefütterten Larven deutete auf suboptimale Ernährungsbedingungen hin, die mit der größten Zahl von hochregulierten Proteinen korrelierte; während die große Körpermasse von pTV*cpn*-gefütterten Larven auf optimale Ernährungsbedingungen verglichen mit pTV*gmps*-gefütterten Larven hindeutete, was mit der kleinsten Anzahl von hochregulierten Proteinen korrelierte. Diese Beobachtungen legen nahe, dass die Inaktivierung von Genen dieser differentiell bei Pflanze-Herbivor-Interaktionen regulierten Proteine und die genaue Analyse der einzelnen Mitspieler in der Interaktion neue Genfunktionen auf der Ebene des Organismus aufdecken können.

III. Der Angriff von Herbivoren reduziert die Gehalte an Photosyntheseproteinen und die photosynthetische Kapazität der Pflanze, während er die Produktion und Anreicherung von Stoffwechselprodukten, die an der Abwehr beteiligt sind, erhöht. *N. attenuata* vermindert nach Insektenfraß die RuBPCase aktivase und RuBPCase, zwei wichtige Enzyme der Photosynthese, um die Verteilung der Ressourcen für Wachstum, Reproduktion und Abwehr zu optimieren. Das Silencing dieser zwei Gene in *N. attenuata* führte zu einer starken Verminderung von Photosynthese und Wachstum. Jedoch sind Pflanzen mit inaktivierter RCA weniger resistent als Pflanzen mit inaktivierter RUB und mit leerem Vektor transformierte Pflanzen. Das Wachstum und die Entwicklung von Herbivoren auf RCA- und RUB-inaktivierten Pflanzen stimmen mit ihrer Anreicherung von Jasmonat-Ile/Leu und Abwehrmetaboliten überein. Die Gesamtphotosynthesekapazität von *N. attenuata* beeinflußt die Verteilung von

Ressourcen, die wiederum Wachstum, die Produktion von Abwehrstoffen und die Resistenz gegenüber Herbivoren beeinflußt.

Die Beschreibung von mehr atypischen Proteinen, die auf Pflanzenfraß reagieren, und die vergleichende Analyse des metabolomischen Profils von transgenen Pflanzen können neue Abwehrstrategien von Pflanzen aufdecken. Darüber hinaus, kann eine unvoreingenommene Transkriptom- und Proteomanalyse von Herbivoren, die auf verschiednen transgenen Linien gefressen haben, Forschern helfen zu entziffern, wie Pflanzenfresser mit neuen Abwehrstrategien von Pflanzen fertig werden.

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8. Acknowledgements

Many thanks to

- Prof. Ian Baldwin for his supervision, ongoing support, and many inspirational ideas and discussions at every stage of the project
- Claudia for helping me to get started
- Hendrik, Ashok, Jorge, Vandana for helping hands and thoughts
- Mass spectrometry group for the analysis of protein sequences
- Susi, Anjte, Klaus, and Thomas for making transformants, VIGS constructs and sequencing
- Mathias, Eva for helping me with LC-MS analysis
- Danny, Beatrice, Rayko, Silvia, and Markus for a great Utah field season in summer 2006
- Paulina and Angelika for helping me with *Spodoptera* larvae
- Karin for all sorts of help and being there
- Evelyn for invaluable help with administrative matters
- My office mates- Jin-Ho, Oz, Siham, Samir, Hendrik, and Vijay – and my colleagues – Anke, Caro, Jianquang, Christian, Stefan, Gowda, Shree, Om, Mery, Paulina, Dalial, Anna, Fabio, Radhika, and Rohit-- for their friendly support, chats and social activities
- Emily for editorial assistance
- All people at the Friedrich-Schiller-University directly or indirectly involved in the dissertation process
- International Max-Planck Research School for financial assistance
- My parents and friends outside the institute for their unremitting support

9. Eigenständigkeitserklärung

Entsprechend der geltenden, mir bekannten Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena erkläre ich, daß ich die vorliegende Dissertation eigenstängig angefertigt und alle von mir benutzten Hilfsmittel und Quellen angegeben habe. Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Fertigstellung der Manuskripte unterstützt haben, sind am Beginn eines jeden Manuskripts genannt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten. Die vorgelegte Dissertation wurde außerdem weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht.

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10. Curriculum vitae

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11. Publications

Giri AP[§], Wünsche H[§], **Mitra S**[§], Zavala JA, Muck A, Svatos A, Baldwin IT (2006) Molecular interactions between the specialist herbivore Manduca sexta (Lepidoptera, Sphingidae) and its natural host Nicotiana attenuata. VII. Changes in the plant's proteome. *Plant Physiology* 142: 1621-1641

[§] These authors contributed equally

Mitra S, Wünsche H, Giri AP, Hivrale V, Baldwin IT (2007) How do plants cope with herbivore attack? Ask the proteome: silencing genes of seven herbivore-responsive proteins reveals their role in plant-herbivore interactions in *Nicotiana attenuata*, in review, *Proceedings of National Academy of the Sciences of the United States of America*.

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12. Appendices

Supplementary Material (CD ROM)

Manuscript I

Table: Primer used for RT-PCR analysis

Figure 1: 2DE analysis of nuclear proteins.

Figure 2: Proteomic maps of two-dimensionally separated control and elicited leaf proteins 6 and 12 h after elicitation.

Figure 3: Proteomic maps of two-dimensionally separated control and elicited leaf proteins 48 and 72 h after elicitation.

Figure 4: Two-dimensional gel analysis of control and elicited leaf proteins.

Figure 5: Location of peptides obtained from protein spots on identified database protein sequence.

Manuscript II

Table 1: Primer used for RT-PCR analysis.

Table 2: The slope of A/Ci curves.

Table 3: Statistical analysis

Table 4: List of differentially regulated proteins in *M. sexta*

Figure 1: Transcript levels of targeted genes after silencing

Figure 2: Phenotype of transformed *N. attenuata* plants

Figure 3: Measurement of photosynthesis (A/Ci curve)

Figure 4: Plant biomass and secondary metabolites accumulation (whole plant)

Figure 5: 2-DE profile of *M. sexta*'s gut proteins

Manuscript III

Figure 1: Southern blot

Figure 2: A/Ci curve